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Approaches to the Genetic Analysis of Mammalian Cells

Approaches to the Genetic Analysis of Mammalian Cells

Michigan Conference on Genetics

oseph, 1921—

Edited by Donald J. Merchant and James V. Neel

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Preface

AMONG the many exciting genetic developments of recent years, none exceeds in implications the advances responsible for the emergence of a variety of approaches to the genetic analysis of mammalian cells maintained in tissue culture. Human cells can now be grown, put in suspension, plated, and cloned much as bacteria. A wide number of genetic traits can be studied at the cellular level, and insight gained into the degree to which secondary homeostatic reactions obscure the picture of primary gene action in mammals. Spontaneous and induced mutation rates can be investigated for selected traits, and the relative significance to man of a variety of mutagenic agents evaluated much more quickly than in population studies. The relationship of variants appearing in the course of culture to chromosomal changes can be defined within the limits of the cytological approach. A number of laboratories are working on transduction and transformation in mammalian cells. Vegetative hybridization between mammalian cells has been reported.

The limiting factor in these and other developments will frequently be the availability of convenient, genetically determined marker traits whose presence can be readily detected under diverse cultural conditions. Moreover, this same limitation blocks the effective application of cell culture methods to a variety of other problems in biology and medicine. The present difficulty in detecting cross contamination of cell lines as well as the more general question of characterizing cells grown *in vitro* are but examples of the practical problems involved.

The essays appearing in this issue are based on lectures presented at The University of Michigan during the spring of 1962 under the title, "Approaches to the Genetic Analysis of Mammalian Cells." This series of lectures stemmed from the conviction that there was a need for summarization of the present state of our knowledge regarding genetic markers for mammalian cells, a conviction which the organizing committee, composed of Rowland H. Davis, William H. Murphy, Francis E. Payne, Margery W. Shaw, and Donald J. Merchant (Chairman), quickly discovered was shared by many workers in the field. The committee and the editors of this issue are grateful to all the speakers, the excellence of whose presentations was matched by the promptness with which their manuscripts were submitted. We are grateful to The University of Michigan MEDICAL BULLETIN and The University of Michigan Press, whose publication timetables were equally exceptional. Finally, the

Lecture Series was possible only because of generous financial support from the Institute of Science and Technology, the Medical School, and the Cancer Research Institute of The University of Michigan.

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Applicability of Approaches of Microbial Genetics to Characterization of Mammalian Cells

FRANCIS J. RYAN, Ph.D.

WHEN Dr. Merchant asked whether I would give this first lecture in the series on "Approaches to the Genetic Analysis of Mammalian Cells," I consented and told him he could invent a title for my talk if he wished. He did. When I read his title I wondered what I could say on the subject. I have worked with bacteria and with fungi and even with fish and amphibians—but never with mammalian cells. In my apprehension, Dr. Merchant's title was transposed in my mind and began to read, "Applicability of approaches of microbial genetics to the characterization of microbial cells." I began in this way to see that the approaches used in microbial genetics could be divided into two major groups.

In the first place certain approaches were directed toward some aspect of the natural history of the microbes themselves. For example, Joshua Lederberg[1] once asked whether bacteria might exchange genes by mating. The answer was *yes,* and considerable detail is now known about the process, especially as a result of the work of Jacob and Wollman.[2] Another instance in which the object of an investigation was the organism itself is the work of Hershey and Chase[3] on bacteriophage. In an attempt to understand the process of infection, they discovered that the phage injected its DNA into the bacterial host. Thereupon followed considerable illumination of the life history of phage. But of even more importance was the solution of basic, pervasive questions, such as what is the chemical basis of inheritance. Without the work of Hershey and some others, we might still be speculating on the way in which protein embodies genetic information and we certainly would be putting much less emphasis on that beautiful three-letter word, DNA.

Another in Hershey's company was Oswald Avery. He and his collaborators[4] also undertook the natural historical approach and tried to find out what it was in the extracts of virulent pneumococcus that transformed sensitive cells. Avery surprised himself by showing it was not the polysaccharide and equally surprised the geneticists by showing it was not protein. With his work began the era we now all recognize as belonging to molecular biology.

Others cooperated to help launch this brave new world of biology, some taking very different approaches. Beadle and Tatum,[5] for instance, were motivated by an idea rather than by an organism. They reckoned that it should be possible to induce mutations for any character they wished to study, even a biochemical one. In searching for subject material they hit upon the red bread mold, *Neurospora;* for them it was a tool rather than an object. So it has been

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with other molecular biologists. Bacteria were used by Meselson and Stahl[6] to determine whether the replication of DNA was semiconservative as Watson and Crick[7] had predicted. Freese[8] and Crick, Barnett, Brenner, and Watts-Tobin[9] used bacteriophage to understand the molecular basis of mutation. They all answered their theoretical questions but, in so doing, better characterized the organisms with which they were working, although this was not their intention.

As a result many microorganisms are better understood than most macroorganisms. This is in part due to the fact that microbes are more easily pushed around experimentally. Their genetic investigation had also long been neglected so that what could be learned from them quickly was a freshening flood of facts and concepts in genetics. Will knowledge gained about the genetics of mammalian cells constitute a comparable breakthrough in the general understanding of genetics, or will they simply relate to mammalian cells themselves? There are those who feel that somatic cell genetics must simply follow in the path of the genetics of microbial cells. I have often felt that way, but upon reflection, it seems that this proposition cannot be entirely true. Mammalian somatic cells are so different from microbes that there must be forthcoming concepts, of pervasive significance for diploid organisms with chromosomes, which most microbes cannot yield.

I would like now to talk about two subjects which have been investigated in my laboratory: the first in reference to a general theoretical problem, the second concerning a situation in the natural history of bacteria. Then I will turn again to the way in which studies of mammalian cells may illuminate more problems than concern the cells themselves.

Some of my students, notably Rudner[10] and Strelzoff,[11] have attempted to see whether bacterial mutations may occur as errors in the course of gene replication. They employed the base analogues, 5-bromouracil (BU) and 2-aminopurine (AP), which are incorporated into DNA in place of thymine (T) and adenine (A), respectively, and which are actively mutagenic. The simplest experiment involved the use of synchronized populations of bacteria exposed to the analogue for only the first replication of DNA. The consequences differ according to whether it is assumed that the mutation involves a transition of a single base pair in the DNA from A:T (adenine:thymine) → G:C (guanine:cytosine) or from G:C → A:T. Figure 1 shows that, providing we are dealing with point mutations, both transitions are completed in three replications. However, in the case of the G:C → A:T transitions, every clone of cells that, rarely, incorporated BU or AP would have given rise to an A:T mutant at least by the third replication. Thereafter no new clones will give rise to induced mutants. In the case of A:T → G:C transitions, the model shows the rare (erroneous) pairing of BU with G (or of AP with C) in the second replication. It might have taken place in the third or any subsequent replication with equal probability. As a consequence, replication by replication, new clones of bacteria, having incorporated BU or AP, will throw off G:C mutants at a constant rate.

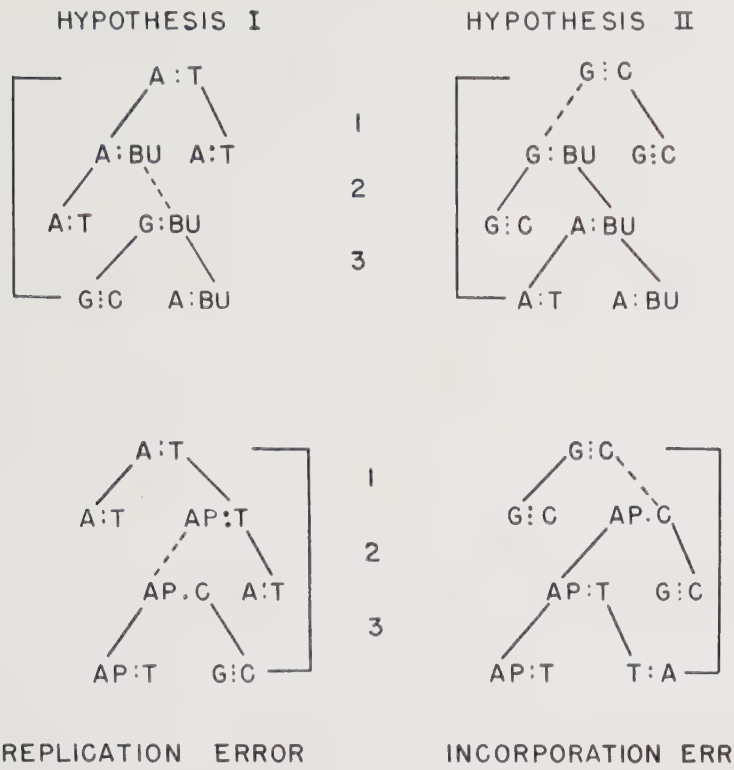

Fig. 1.—Models for the mutagenic action of incorporated base analogues on the assumption that they induce a transition of one base pair into another at some site in the DNA.

Experimentally it was found that amino acid-requiring auxotrophs reverted in response to these base analogues in one or the other pattern; either all the induced mutations were found by the third replication or the induced mutations continued to appear, replication by replication, at a constant rate (Figure 2). Incorporation errors of the first sort indicated that the mutation involved a transition from G:C → A:T; replication errors of the second sort spoke for a transition from A:T → G:C. Is there anything about mammalian somatic cells that would have prevented such an identification of the molecular basis of mutation in them? Or was it achieved with bacteria only because of an attitude of mind? I favor the latter interpretation.

Now let me turn to a question concerning the natural history of bacteria. Most people are not surprised that a stock of bacteria kept by serial transfer in a laboratory retains its characteristics for years and years. But they should be! Bacteria are haploid organisms which reproduce clonally. As mutation occurs among them they should become phenotypically as well as genotypically heterogeneous. Eventually each gene should be represented in the population with its alleles in mutational equilibrium with each other. The best approximations allow calculations showing that only an insignificant fraction

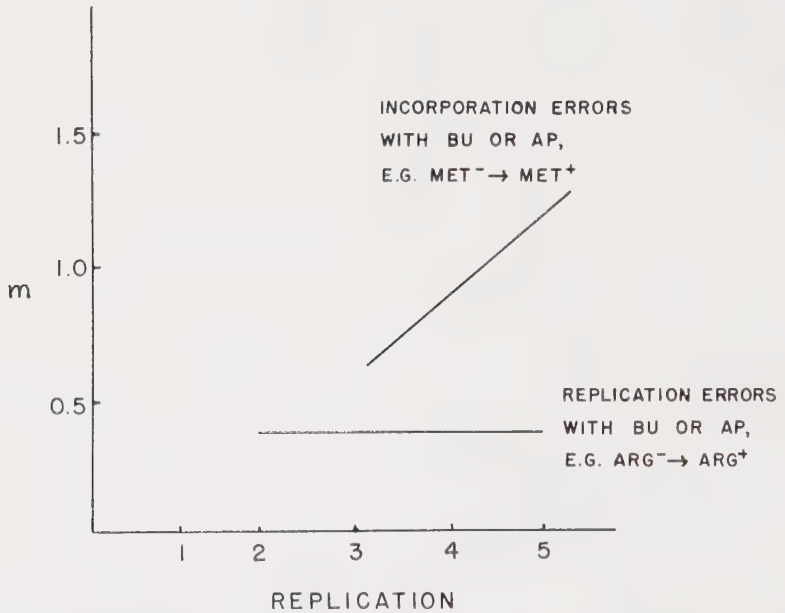

Fig. 2.—The pattern of appearance of reversions to prototrophy in different auxotrophs of *E. coli* (Strelzoff, 1962[11]). The ordinate records the average number of mutations per culture (m) and the abscissa the number of replications the synchronized population has undergone, including the first in the presence of a base analogue (BU or AP).

of the population would be alike. Yet this is not so; pure strains remain pure with only very low proportions of variant types.

Anyone who has worked with populations of cells, whether they be bacterial or mammalian, knows that there is something wrong with the assumptions just made. Every given medium is a selective agent; every kind of cell is not equally well adapted to every medium. Therefore, the medium tends to fix a given character in a strain by favoring those which possess it. Media containing streptomycin, for example, will select against streptomycin-sensitive cells and fix streptomycin-resistant and streptomycin-dependent characters in the population. It seems illogical, however, to imagine that the same medium would fix alternate characters, say streptomycin sensitivity and streptomycin resistance, but this is in fact what happens.

A streptomycin-sensitive culture remains that on nutrient broth and is not overgrown by resistant mutants; however, the same is true of a streptomycin-resistant strain which is not overgrown by sensitive mutants. This is true whatever the pair of phenotypic characters; there is a neutral medium on which both strains will remain stable. This means that there are at least two equilibrium levels for any mutant type—one very high for the strain said to be of that type and one very low for the strain said to be its alternate.

How can there be two equilibrium levels for a given character? The answer is found in a phenomenon called orthoselection or periodic selection.[12–15] It

is based upon the fact that mutations of many sorts continually take place which confer a superior adaptation to the environment upon the mutant. Since these mutant genes are not spread among neighboring bacteria but are given only to the clone of descendants of the original mutant, the fitter clone overgrows the others in its environment. As a consequence there is a progressive replacement of types in a continuously growing population, for example:

$$a \rightarrow b \rightarrow c \rightarrow \rightarrow n$$

The changes that can be observed in bacterial populations are very subtle, not at all as drastic as the changes observed in tissue cultures when the karyotype is observed to vary. In bacteria the only difference that may be observed between two strains, isolated at successive times from a continually growing population, may be that the last will overgrow the earlier one when they are cultured together. Such an experiment involves, of course, the use of genetic markers to distinguish members of the fitter from members of the less fit strain when they grow together. This is achieved in the following way. The continuously growing population may consist of auxotrophs requiring, say, histidine. They continue to throw off histidine-independent mutants. The reconstruction experiments can be made, therefore, between *his*$^-$ of strain *a* and *his*$^+$ of strain *b,* or vice versa. If the cells of strain *b,* as is the case, invariably overgrow those of strain *a* whatever the marker, then we know that the markers are neutral, without influence on the selection observed. We may conclude that strain *b* is superior to strain *a* in mixture and thereby understand the succession of types observed in the continuously growing culture. Successions of fitter types have been observed to occur, in the longest studied case, for more than 7,000 generations, the last type at any time in the life history of the culture being superior to anything that preceded it.

The progressive development of fitness is an example of the selection of mutants in a fixed environment to which the original type was already adapted. But this selection does not affect all characters. Despite its occurrence, a stock of lactose-fermenting or histidine-independent bacteria will remain stable on a given medium; on the same medium a stock of lactose-nonfermenting or histidine-requiring bacteria will also remain stable. This results from the fact that, while the mutants are increasing within a culture because of mutation pressure, a fitter type arises within the major nonmutant segment of the culture and overgrows everything previously present including the mutants. Mutants then begin to appear among the new fitter component but each time they must begin their rise again, never adding to a previously existing population. For example:

$$\begin{array}{ccccccccc} a\ his^- & \rightarrow & b\ his^- & \rightarrow & c\ his^- & \rightarrow & \rightarrow & n\ his^- \\ \downarrow & & \downarrow & & \downarrow & & & \downarrow \\ a\ his^+ & & b\ his^+ & & c\ his^+ & & & n\ his^+ \end{array}$$

$$\begin{array}{ccccccccc} a\ his^+ & \rightarrow & b\ his^+ & \rightarrow & c\ his^+ & \rightarrow & \rightarrow & n\ his^+ \\ \downarrow & & \downarrow & & \downarrow & & & \downarrow \\ a\ his^- & & b\ his^- & & c\ his^- & & & n\ his^- \end{array}$$

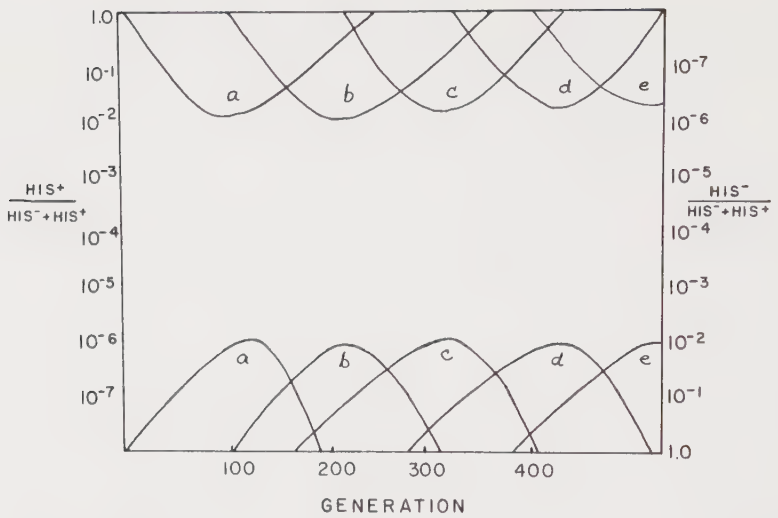

Fig. 3.—Model diagram of the stabilizing effect of orthoselection on the frequency of minority mutant types in cell populations. The lower set of curves is for a culture with *his*⁻ bacteria and represents the course of increase of *his*⁺ mutants among each fitness type of *his*⁻ parent (a,b,c—n) and the course of decrease as a succeeding fitness type of *his*⁻ overgrows the population and begins to throw off *his*⁺ mutants of its own type. The upper curves are equivalent, but represent the increase of *his*⁻ mutants in a culture started with *his*⁺ bacteria. The two equilibria represented for the *his*⁺ cells are ca. 10^{-6} in the *his*⁻ population and ca. $1 - 10^{-6}$ in the *his*⁺ population.

Thus, there will be two equilibrium concentrations of *his*⁺ bacteria depending upon whether the culture began with mostly *his*⁻ or *his*⁺ cells in the first place (Figure 3).

This situation is formally comparable to one in which a small sample is taken from a large population in which mutants are rare, in order to establish a new large population from which a similar sample is taken, and so on. As long as the mutants are rare enough so there is not a large chance that they will be included in the sample (say, in a frequency of 10^{-6} so that only once in a hundred thousand samples of 10 cells will they be included), the sample will consist only of nonmutant cells. As a consequence each sampling destroys whatever mutant rise occurred and each sample's growth involves the same abortive initiation of an increase in frequency. Cultures may be kept "pure" in this way, but they are "purified" in any event by orthoselection which does the same thing. Nevertheless, the selective improvement of the culture cannot be prevented unless the environment is changed. Of course, orthoselection and periodic sampling by other means can be completely overridden by a drastic change in the environment which results in an explosive selection such as that exercised against *his*⁻ bacteria by the absence of histidine or against streptomycin-sensitive bacteria by the presence of streptomycin.

The examination, then, of the population dynamics of bacteria has clearly characterized their behavior in terms of a phenomenon not previously

recognized. It seems inevitable that the phenomenon must be general for clonally reproducing cells, whether diploid or not. Yet it has not been demonstrated outside of bacteria. Mammalian cells can be grown continuously for long times even in a dispersed state and the frequencies of some mutant types among them can be determined. Does orthoselection transcend the barriers of kingdoms? It must, for it depends only upon the facts of random mutability and clonal growth, and these are common denominators among cells. Perhaps the phenomenon will be found in stabilized lines of mammalian cells whose establishment clearly involves a more drastic selection. To demonstrate orthoselection among mammalian cells would only follow a precedent in microbial genetics. However, much work with mammalian cells has done only that and the title of my talk says I should show the ways in which to continue this self-conscious pursuit.

It has been shown that somatic cells mutate; in fact, methods have been developed to concentrate auxotrophs[16] and enzymatic defects have been characterized.[17–19] It has been shown, furthermore, that mutations occur in a random way, [20,21] and the rates of mutation have even been measured with some reliability.[21–24]

Evidently the technical obstacles are surmountable. Most of this series of lectures being held here at Michigan will be concerned with the various inherited traits that may be used as genetic markers of mammalian cells. This is very important; good markers in profusion are a prerequisite for meaningful genetic study. It is also imperative that means of recombination be available. Mutation and recombination analysis have powers of resolution that lie at the level of nucleotide pairs in DNA[25,26]; chemical analyses only now begin to approach that level. Professor Ephrussi will, at the termination of this series, discuss the mating of cells in tissue culture,[27] but claims of success in transforming mammalian cells in tissue culture seem now more valid than before.[28] The use of such techniques should give us information about closely linked human genes and their fine structure which cannot be obtained from uncontrolled human matings. This knowledge could be used in conjunction with the extensive information available about human biochemistry. Should we expect from such studies only an improvement in our concepts regarding mammalian cells? How can studies of mammalian cells illuminate general biological problems and, in so doing, give a most profound and relevant characterization of themselves?

A good deal may be learned about animal viruses and their control from a study of such cells. But can we expect control over abnormal growth without a more profound knowledge of differentiation? Will we, or can we expect to, get this information from a study of microorganisms? Will studies on mammalian cells simply show that this borrowed knowledge is applicable? At the present time studies are being made with microorganisms which promise avenues of approach to cellular differentiation at the level of molecular biology. As a matter of fact, if one reads John Osmundsen's articles in *The New York Times* (January 2, and February 2, 1962), he can confirm the impression that,

now that the broad brush strokes of the picture of gene structure, replication, mutability, and action have been drawn, molecular genetics will concern itself with differentiation.

Clearly we must know how genes are turned on and off, how their action is regulated and coordinated. In dipteran cells this process has been characterized in the form of the puffs of polytene chromosomes.[29] In bacteria, new kinds of genes have been identified.[30] There are structural genes whose triplet (?) sequences specify the sequence of amino acids in proteins through the intervention of messenger RNA. But there are also controlling genes (R), which produce a repressor of O, the operator gene. The state of the operator gene determines whether a series of structural genes make messenger RNA or not; the operator and the series of structural genes it controls constitute the operon, a coordinated unit of function which must be in *cis* conformation. This is undoubtedly the reason why genes in bacteria concerned with steps in the synthesis of a single substance are linked in the order of that synthesis.[31] In "higher" organisms such sequential linkages of genes are not conspicuous; are there operators for single structural genes scattered with them about the chromosomes? More recently it has become evident that there are super-operator genes which control several operons located over quite some length of chromosome.[32–35] What are the laws which determine how these regulatory systems act? Genetic changes within O and R genes modify their behavior so that control may be rigid or relaxed. How do these genes respond to their intracellular environments? Inducers of enzyme synthesis seem to inhibit the action of repressors so that operators under their control are free to act. Are all environmental effects on regulatory systems reversible in this way or can the environment bring about irreversible changes such as those which occur in differentiation?[36]

For how long will microorganisms be the main source of information and ideas about such problems? I suppose it will only be so long as those who work with somatic cells look for guidance and until, with the spirit of optimism and adventure that is beginning to pervade biology these days, they tackle *problems* in their material or with their material undertake the solution of general problems. Microbial geneticists are sensitive to what is trivial; in their large competitive haste they try hard to abide by Gerber's law which says, "What is not worth while doing, is not worth while doing well."

Of course there are technical difficulties and they have not yet all been overcome. The most important methods to be developed for the exploitation of mammalian cell genetics are, in my opinion, those which will allow study of the recombination of genes. Sorieul and Ephrussi[27] have discovered evidences of mating; whether mitotic crossing over or nondisjunction[26] occurs is yet to be seen. Claims of success in transforming somatic cells have been made.[28,37] It should be borne in mind, however, that transformations are hereditary changes; it is, therefore, necessary to show stability of the transformed trait through many generations of cell growth.[37] Briefer modifications, usually achieved with RNA,[38,39] may be involved in differentiation but they are not

transformations.[40] Further study and confirmation of claims for transformation are necessary. The techniques of microbial genetics are well known[41]—mutation, selection, mating, virus infection, transduction by phage and other episomes, transformation, complementation, etc. I expect a heyday in the study of mammalian cells when the ablest of our young investigators involve themselves in the field and, without self-consciousness, rely upon themselves for the development of novel and relevant approaches.

REFERENCES

1. Lederberg, J.: Gene Recombination and Linked Segregations in *Escherichia coli*. Genetics *32*:505–525, 1947.
2. Jacob, F., and Wollman, E.: *Sexuality and the Genetics of Bacteria*. New York: Academic Press, 1961.
3. Hershey, A., and Chase, M.: Independent Functions of Viral Protein and Nucleic Acid in Growth of Bacteriophage. J. Gen. Physiol. *36*:39–56, 1952.
4. Avery, O. T.; McLeod, C. M.; and McCarthy, M.: Studies on the Chemical Nature of the Substance Inducing Transformation of Pneumococcal Types. J. Exp. M. *79*:137–158, 1944.
5. Beadle, G. W., and Tatum, E. L.: Genetic Control of Biochemical Reactions in *Neurospora*. Proc. Nat. Acad. Sc. *27*:499–506, 1941.
6. Meselson, M., and Stahl, F.: The Replication of DNA in *Escherichia coli*. Proc. Nat. Acad. Sc. *44*:671–682, 1958.
7. Watson, J. D., and Crick, F. H. C.: A Structure for Desoxyribose Nucleic Acid. Nature *171*:737–738, 1953.
8. Freese, E.: The Molecular Mechanisms of Mutations. Proc. V Internat. Cong. Biochem. London: Pergamon Press, 1961.
9. Crick, F. H. C.; Barnett, L.; Brenner, S.; and Watts-Tobin, R. J.: General Nature of the Genetic Code for Proteins. Nature *192*:1227–1232, 1961.
10. Rudner, R.: Mutation as an Error in Base Pairing. Zschr. Vererb. *92*:336–379, 1961.
11. Strelzoff, E.: DNA Synthesis and Induced Mutations in the Presence of 5-Bromouracil. Zschr. Vererb. *93*:287–318, 1962.
12. Atwood, K. C.; Schneider, L. K.; and Ryan, F. J.: Periodic Selection in *Escherichia coli*. Proc. Nat. Acad. Sc. *37*:146–155, 1951.
13. Atwood, K. C.; Schneider, L. K.; and Ryan, F. J.: Selective Mechanisms in Bacteria. Cold Spring Harbor Sympos. Quant. Biol. *16*:345–355, 1951.
14. Ryan, F. J.: Natural Selection in Bacterial Populations. Atti VI Cong. Internat. Microb., Roma *1*:649–657, 1953.
15. Ryan, F. J.: Analysis of Populations of Mutating Bacteria. In Kempthorne, O.; Bancroft, T. A.; Gowen, J. E.; and Lush, J. L. (Eds.): *Mathematics and Statistics in Biology*. Ames, Iowa: Iowa State College Press, 1954, chap. 15.
16. DeMars, R., and Hooper, J. L.: A Method of Selecting for Auxotrophic Mutants of HeLa Cells. J. Exp. M. *111*:559–572, 1960.
17. Krooth, R. S., and Weinberg, A.: Properties of Galactosemic Cells in Culture. Biochem. Biophys. Res. Commun. *3*:518–524, 1960.
18. Szybalski, W.; Szybalska, E. H.; and Ragni, G.: Genetic Studies with Human Cell Lines. National Cancer Institute Monograph 7, 1962, pp. 75–88.
19. Paigen, K.: The Genetic Control of Enzyme Activity During Differentiation. Proc. Nat. Acad. Sc. *47*:1641–1649, 1961.
20. Law, L. W.: Origin of Resistance of Leukaemic Cells to Folic Acid Antagonists. Nature *169*:628–629, 1952.
21. Lieberman, I., and Ove, P.: Estimation of Mutation Rates with Mammalian Cells in Culture. Proc. Nat. Acad. Sc. *45*:872–877, 1959.
22. Atwood, K. C., and Scheinberg, S. L.: Somatic Variation in Human Erythrocyte Antigens. J. Cellul. Physiol. *52*(Suppl.):97–123, 1958.
23. Atwood, K. C.: The Presence of A_2 Erythrocytes in A_1 Blood. Proc. Nat. Acad. Sc. *44*:1054–1057, 1958.

24. Szybalski, W.: Genetics of Human Cell Lines; II. Method for Determination of Mutation Rates to Drug Resistance. Exp. Cell Res. *18*:588–591, 1961.
25. Benzer, S.: The Fine Structure of a Gene. Sc. Am. *206*:70–84, 1961.
26. Pontecorvo, G.: *Trends in Genetic Analysis*. New York: Columbia University Press, 1958.
27. Sorieul, S., and Ephrussi, B.: Karyological Demonstration of Hybridization of Mammalian Cells *in vitro*. Nature *190*:653–654, 1961.
28. Szybalski, W., and Joannes, S. A.: Genetic Transformation in Human Cells. Microb. Genetics Bull. *18*:22–23, 1962.
29. Beermann, W.: Nuclear Differentiation and Functional Morphology of Chromosomes. Cold Spring Harbor Sympos. Quant. Biol. *21*:217–232, 1956.
30. Jacob, F., and Monod, J.: Genetic Regulatory Mechanisms in the Synthesis of Proteins. J. Mol. Biol. *3*:318–356, 1961.
31. Demerec, M., and Hartman, P. E.: Complex Loci in Microorganisms. Annual Rev. Microb. *13*:377–406, 1959.
32. McClintock, B.: Some Parallels Between Gene Control Systems in Maize and in Bacteria. Am. Naturalist *95*:265–277, 1961.
33. Revel, H. R.; Luria, S. E.; and Rolman, B.: Biosynthesis of β-D-Galactosidase Controlled by Phage-Carried Genes; I. Induced β-D-Galactosidase Biosynthesis after Transduction of Gene Z-plus by Phage. Proc. Nat. Acad. Sc. *47*:1956–1967, 1961.
34. Revel, H. R., and Luria, S. E.: Biosynthesis of β-D-Galactosidase Controlled by Phage-Carried Genes; II. The Behavior of Phage-Transduced Z-plus Genes Toward Regulatory Mechanisms. Proc. Nat. Acad. Sc. *47*:1968–1973, 1961.
35. Revel, H. R.; Luria, S. E.; and Young, N. L.: Biosynthesis of β-D-Galactosidase Controlled by Phage-Carried Genes; III. Derepression of β-D-Galactosidase Synthesis Following Induction of Phage Development in Lysogenic Bacteria. Proc. Nat. Acad. Sc. *47*:1974–1980, 1961.
36. Moore, J. A.: Nuclear Transfer of Embryonic Cells of the Amphibia. In Walker, P.M.B. (Ed.): *New Approaches in Cell Biology*. New York: Academic Press, 1960, pp. 1–14.
37. Kraus, L.: Formation of Different Haemoglobins in Tissue Culture of Human Bone Marrow Treated with Human DNA. Nature *192*:1055–1057, 1961.
38. Niu, M. C.; Cordova, C. C.; and Niu, L. C.: Ribonucleic Acid-Induced Changes in Mammalian Cells. Proc. Nat. Acad. Sc. *47*:1689–1700, 1961.
39. Ranzi, S.; Gavarosi, G.; and Citterio, P.: Cytodifferentiation Induced by Ribonucleoproteins. Experientia, Basel *17*:395–396, 1961.
40. Shen, S. C.; Hong, M. M.; Cai, R. Z.; Chen, H. Z.; and Zhang, W. L.: Ribonucleic Acid as a Transforming Principle in Bacteria. Proc. V Internat. Cong. Biochem. London: Pergamon Press, 1961, p. 409.
41. Sager, R., and Ryan, F. J.: *Cell Heredity*. New York: John Wiley & Sons, 1961.

Drug Sensitivity as a Genetic Marker for Human Cell Lines

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WHEN we began our part-time venture into the genetic exploration of human tissue cultures some three or four years ago, I realized neither that I would be speaking here about that topic, nor that my talk would be preceded by a lecture of Dr. Ryan entitled "Applicability of Approaches of Microbial Genetics to Characterization of Mammalian Cells."[1] Biased, however, by the common background of microbial genetics, we followed along the same line advocated by Dr. Ryan. In addition, I must make one more confession: we never abandoned bacterial genetics, and at present human tissue cultures and their potential contaminants, *Escherichia coli* and *Bacillus subtilis,* flourish in adjoining rooms of our laboratory. Actually, the choice of a given organism as best suited for a particular problem is only incidental, the solution of the general biological problem as such being the primary concern. There are some problems for which the tissue culture cell would appear to be the organism of choice, particularly now that the quantitative methods of cloning and colony scoring, pioneered by T. T. Puck,[2] have become generally applicable, and since several selective mutational systems have been developed. In this presentation I plan to deal mainly with the latter systems and some of their applications.

CHOICE OF THE STANDARD CELL LINE

At the outset of our studies the decision was reached to concentrate on only one cell line, preferably of nonmalignant human origin. We reasoned that the choice of a line with favorable cultural characteristics, including high plating efficiency, and with genetic and chromosomal stability would facilitate quantitation of future genetic studies and thus assure their meaningfulness. Ideally, these characteristics should permit an ease of manipulation comparable to that experienced with *E. coli* strains B or K12. While the theoretical advantages of euploidy were appreciated, the practical difficulties connected with maintaining cells in this state *in vitro* ruled out their use at the time. Concerning the application of the present studies to human genetics and medical problems, past experience has indicated that had the choice of bacterial strain been based on pathogenicity rather than ease of manipulation, the explosive progress in microbial genetics and its early application to medical problems would not have resulted.

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With this justification in mind and only after a thorough survey of the available cell lines, we settled on the Detroit 98 strain[3] as our standard "work horse," a line originally derived from human sternal puncture. After repeated subcultivation and recloning in modified[4] Eagle's medium supplemented with 10 percent horse serum (E_{90} medium), a single clone-derived subline was selected on the basis of its capacity to form compact and uniform colonies with high efficiency. This subline, denoted D98S, proved to be very stable under standard growth conditions, retaining a mean chromosome number of 63 throughout the period of study.[5] The compact character of the colonies

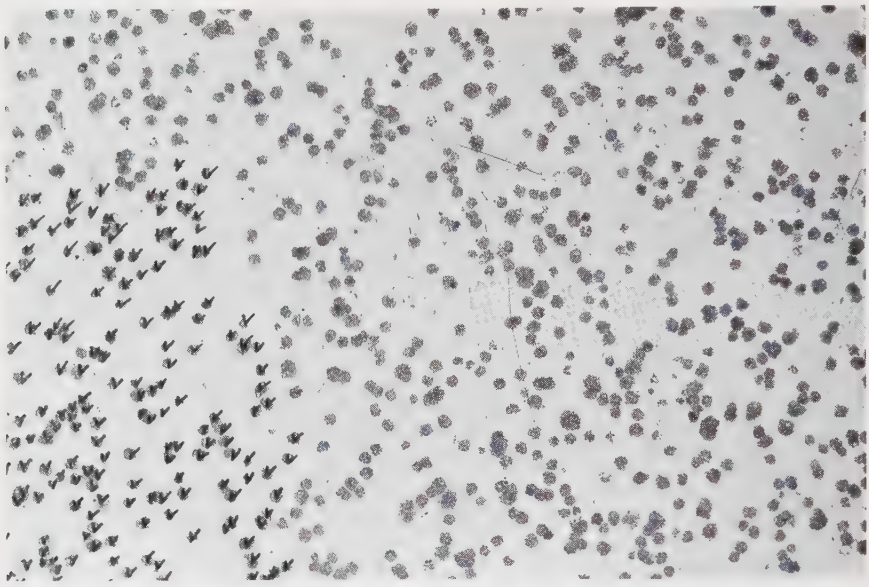


Fig. 1.—The projected image of methylene blue-stained colonies formed 7 days after seeding of a 60 mm. dish with 5,000 D98S cells and incubation in a 5 percent CO_2 atmosphere at 36°C. in Eagle's medium supplemented with 10 percent horse serum. Checking off the colonies with a pencil results in automatic scoring of the colonies by an electronic counter coupled to the pencil (cf. Figure 2).

(Figure 1) permits scoring up to 10,000 clones per 60 mm. plate, using a projection and semiautomatic counting procedure (Figure 2).

All the mutant lines described here were derived from the D98S strain. The media and procedures for cultivation, plating, and colony counting were described earlier.[4-6]

SELECTION OF GENETIC MARKERS

As advocated by Dr. Ryan in his introductory lecture,[1] "development of good genetic markers in profusion is a prerequisite for meaningful genetic studies." We have concentrated on this task since 1958, rejecting all but "good" marked strains. What are "good" selective markers? The criteria

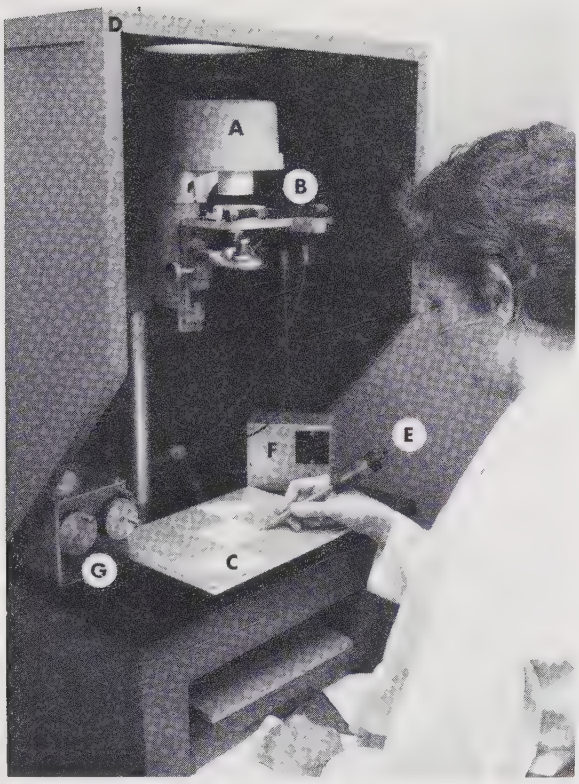


Fig. 2.—Projecting microscope "A" (Bausch & Lomb "Trisimplex") adapted for counting up to 10,000 stained colonies per 60 mm. plate (objective, 1.5×) or individual cells (objective, 5× or 10×). For plates "B" containing over 500 colonies special masks are employed, which permit screening only one tenth of the plate surface (cf. the photograph of the dish image "C"). The portable housing "D" permits counting in a lighted room. Marking the colony images with the switch-operating pencil "E" results in automatic scoring of the colonies on the electronic counter "F." A device "G" is provided for remote scanning of the whole dish.

should include: (a) all or none response of the strain to the particular selective growth conditions; (b) unimpaired growth rate, plating efficiency, and other cultural properties under standard and selective growth conditions; (c) genetic stability even under nonselective growth conditions.

Although chromosomal and certain other morphological markers could find accessory use in genetic studies, good selective characters such as complete nutritional dependence versus independence, or high-level resistance to a drug, virus, or antiserum versus sensitivity, are obviously the markers of choice.

Early in the course of the study, we decided to look for resistance to known antineoplastic drugs, biased both by previous experience[7,8] with bacterial resistance markers and by the familiar tendency of malignancies to acquire drug resistance *in vivo*. Our hopes were first realized by the isolation of a mutant line D98/AG, which in one step had acquired approximately one hundred fold resistance to 8-azaguanine (AG). Our 1958 report[9] described this highly selec-

tive genetic marker in a human cell line, endowed with all the characteristics of a "good" marker.

During the following three years (1959 to 1961), several other examples of *in vitro* resistance development to purine analogues by mammalian cell lines were described in the literature.[4,10–15]

Since the subject of my lecture is drug resistance as a genetic marker, I shall try to develop this theme, using the example of the sequential selection of mutants with increased resistance or sensitivity to analogues of guanine or hypoxanthine and their nucleosides (Figure 3). It is interesting to note that

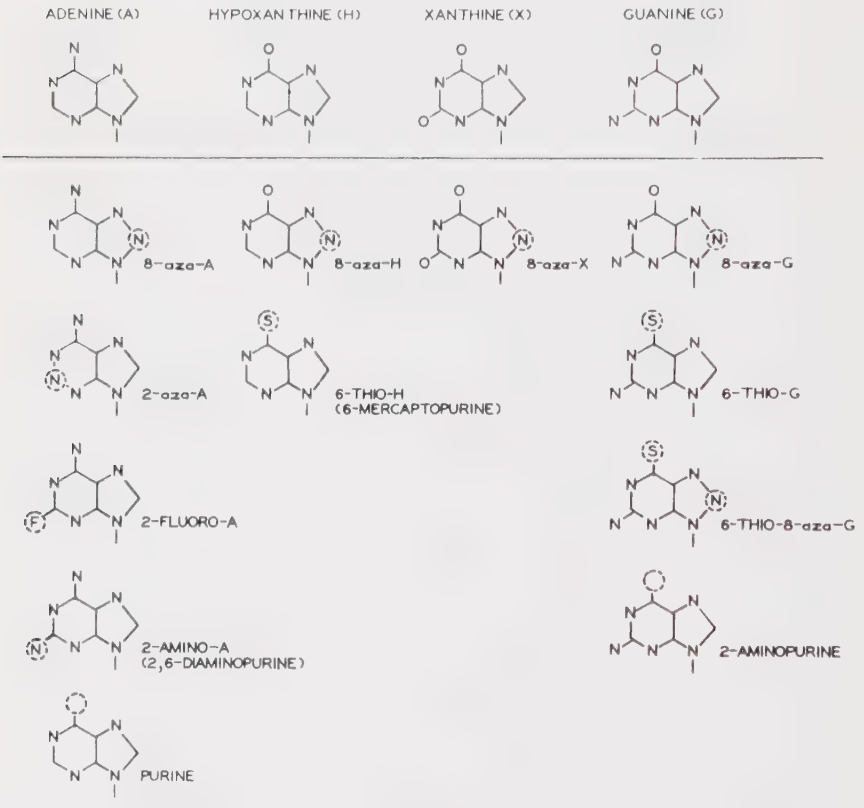


Fig. 3.—Chemical structures of naturally occurring purine bases and their analogues.

all these analogues, when compared with the natural purines, are characterized by *only one* atomic modification, mainly an N for C or S for O substitution. Some analogues with two such substitutions were tested (e.g. 8-aza,6-thioguanine) and found to be completely inactive as inhibitors. Apparently such analogues have lost affinity for the target enzymes. The derivation of the mutant lines is illustrated in Figure 4, and some of their characteristics are summarized in Table 1.

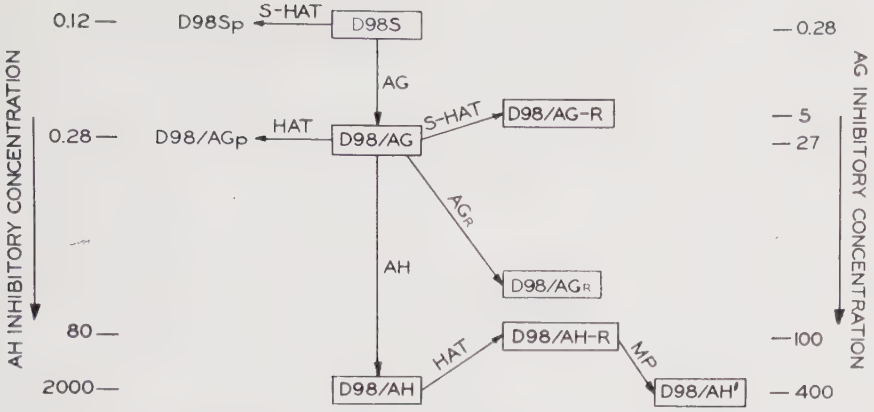


Fig. 4.—Origin of mutant lines sequentially derived from the wild-type D98S. The properties of the mutants are described in the text (including Table 1) and graphically presented in Figures 5, 6, 7, 8, and 10.

1. Selection of Drug-Resistant Clones.—When plated in graded concentrations of 8-azaguanine (AG), up to a level of about 0.1 μg./ml., D98S cells are able to form colonies with normal plating efficiency (Figure 5). Above this concentration the percent survival rapidly decreases, and then stabilizes at approximately 1 to 2 percent over the concentration range of 1 to 10 μg.

TABLE 1.—*Relative Utilization of Natural Purines and Their Analogues (Cross Resistance Factor) in Resistant Mutants (as Compared with the Wild-Type Strain D98Sp)*

Purines and Purine Analogues	Strain					
	D98Sp*	D98/AG	D98/AG-R	D98/AH	D98/AH-R1	D98/AH'
Hypoxanthine†	1× (0.08)	6 ×	1×	> 6000 ×	1 ×	> 6000×
Inosine†	1× (0.12)	7 ×		1700 ×	1.3×	
Inosinic acid†	1× (0.23)	5 ×		3000 ×	1.1×	
Adenine†	1× (0.06)	2 ×		0.7×	1.3×	
8-azaguanine	1× (0.12)	100 ×	50×	3300 ×	1000 ×	3000×
6-thioguanine	1× (0.006)	20 ×		2000 ×	1400 ×	2500×
8-azahypoxanthine	1× (0.12)	2.3×		15000 ×	180 ×	15000×
6-mercaptopurine	1× (0.06)	3 ×		3600 ×	2 ×	3500×
8-azaadenine	1× (0.6)	1.3×		1.3×	0.2×	
2-fluoroadenine	1× (0.002)	1.3×		0.6×		
2,6-diaminopurine	1× (0.4)	2 ×		1 ×	0.5×	
Purine	1× (0.1)	1.5×		2 ×		

* The figures in parentheses represent the concentrations (μg./ml.) permitting 50 percent of the normal plating efficiency either in AT medium (natural purines) or in E_{90} medium (purine analogues) for the D98Sp strain. The values followed by the × sign in the other columns represent the factors by which these concentrations are increased or decreased in the mutant strains as compared with D98Sp(1×).

† Assayed in E_{90} medium containing 0.02 μg. aminopterin/ml. and 5 μg. thymidine/ml. (AT medium).

FIG. 5

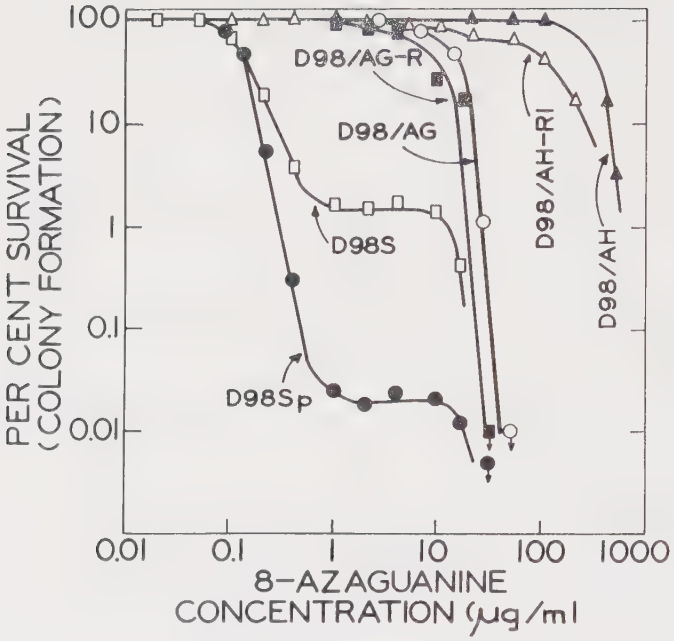
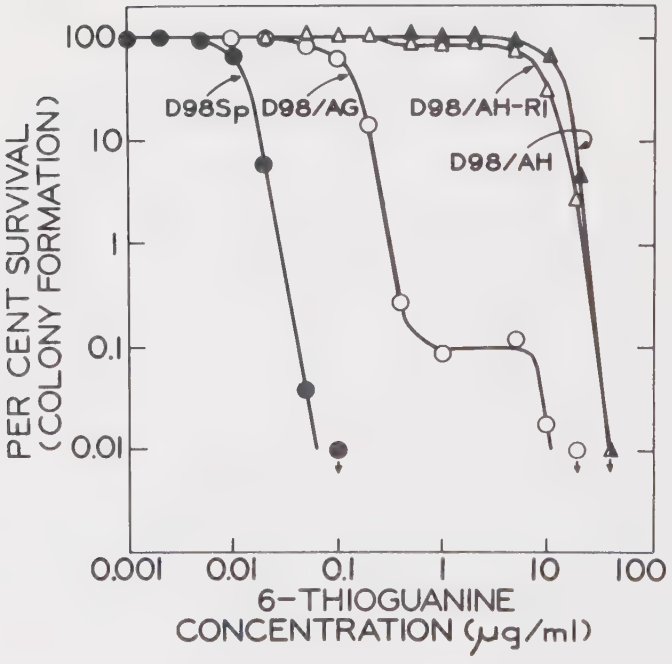
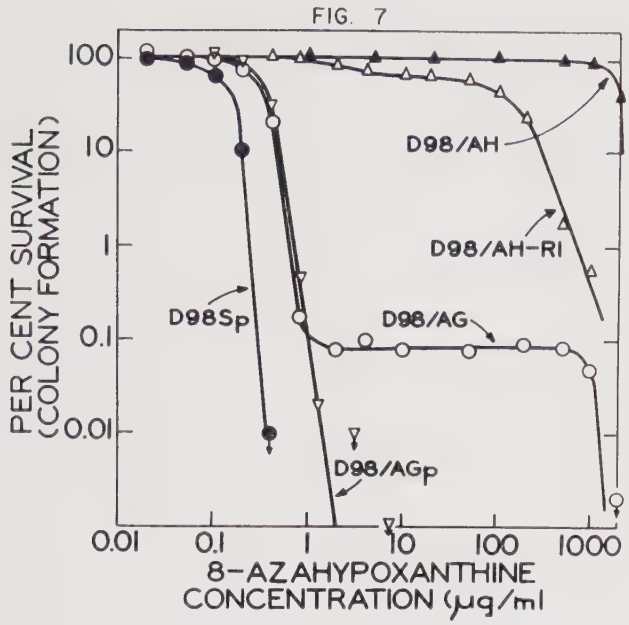

100
10
1
0.1
0.01
PER CENT SURVIVAL
(COLONY FORMATION)
D98/AG-R
D98/AG
D98S
D98Sp
D98/AH-RI
D98/AH
0.01
0.1
1
10
100
1000
8-AZAGUANINE
CONCENTRATION (µg/ml)

FIG. 6


100
10
1
0.1
0.01
PER CENT SURVIVAL
(COLONY FORMATION)
D98Sp
D98/AG
D98/AH-RI
D98/AH
0.001
0.01
0.1
1
10
100
6-THIOGUANINE
CONCENTRATION (µg/ml)



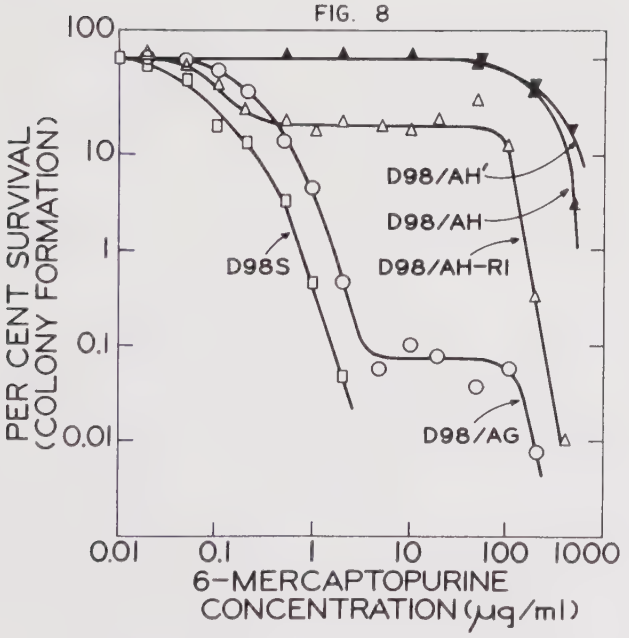


Fig. 5-8.—Survival, expressed as colony formation (percent of the control on E_{90} medium), of the cloned human cell line D98S, its subline D98Sp "purified" on S-HAT medium (cf.: methods for isolation of "purified" mutant stocks), and of several mutant lines, the origins of which are shown in Figure 4, in the presence of increasing concentrations of 8-azaguanine (AG), 6-thioguanine (TG), 8-azahypoxanthine (AH), and 6-mercaptopurine (MP).

AG/ml. At still higher AG concentrations, there are no survivors. Cell lines developed from single clones growing at 1 to 10 μg. AG/ml. were found to plate with normal efficiency at AG concentrations up to 10 to 20 μg./ml. The AG-resistant mutant line designated D98/AG was found to retain its one hundred fold AG resistance upon long-term subcultivation in the absence of AG. The chromosome number and the cultural characteristics of the D98/AG line (and of the other lines shown in Figure 4) do not differ from those of the wild-type D98S.

The resistance of the D98/AG line to the other guanine analogue, 6-thioguanine (TG), was increased by a factor of 20 (Figure 6), while resistance to analogues of hypoxanthine and adenine was increased by threefold or less (Table 1). It might also be considered of some interest that, when compared with D98S, the D98/AG mutant is threefold more resistant to the pyrimidine analogue, 5 fluorouracil (FU),[4] although the mechanisms of action of FU and AG would seem to have nothing in common.

Plating of large inocula of D98/AG in the presence of 8-azahypoxanthine (AH) at concentrations above 5 μg./ml. led to the isolation of another mutant line, D98/AH, which proved to be highly resistant to this analogue (Figure 7).* The seven thousand fold increase in AH resistance of the D98/AH line, as compared with its D98/AG parent, is accompanied by twelve hundred fold cross resistance to 6-mercaptopurine (MP), another hypoxanthine analogue (Figure 8), and lesser increases in resistance to TG (one hundred fold) and AG (thirty-three fold) (Table 1).

2. Mechanism of Drug Resistance.—A cooperative effort with Dr. R. W. Brockman of the Southern Research Institute was aimed at determining the biochemical basis of resistance to purine analogues in these mutant lines. As represented in Figure 9, the analogue must first (a) enter the cell, and then (b) be converted into the active nucleotide form by the action of the appropriate pyrophosphorylase. Steps (a) and (b) thus represent targets for possible genetic blocks. While the second-step mutation, D98/AG to D98/AH, represents essentially total elimination of inosinic-guanylic acid pyrophosphorylase activity, i.e. a block in step (b), the mechanism of the D98S to D98/AG mutation does not appear to involve pyrophosphorylase activity, although the AG to AG-nucleotide conversion by intact D98/AG cells is highly depressed. Thus the genetic block in utilization of AG by the D98/AG strain apparently involves some reaction (step *a*) prior to ribophosphorylation. This deficiency would be amplified by the action of the AG-inactivating enzyme, guanase, present in all the D98 lines.

The results of these experiments were summarized earlier[16] and will be described in more detail elsewhere.

* Strain D98/AG is only threefold more resistant to 8-azaguanosine (AGR) than the D98S line. By plating at inhibitory levels of AGR, the D98/AGR strain was isolated, which was 60 times more resistant to AGR than D98/AG but only 4 times more resistant to AH. This mutant (see Figure 4), which was described earlier,[5] has not been included in the present discussion for the sake of brevity.

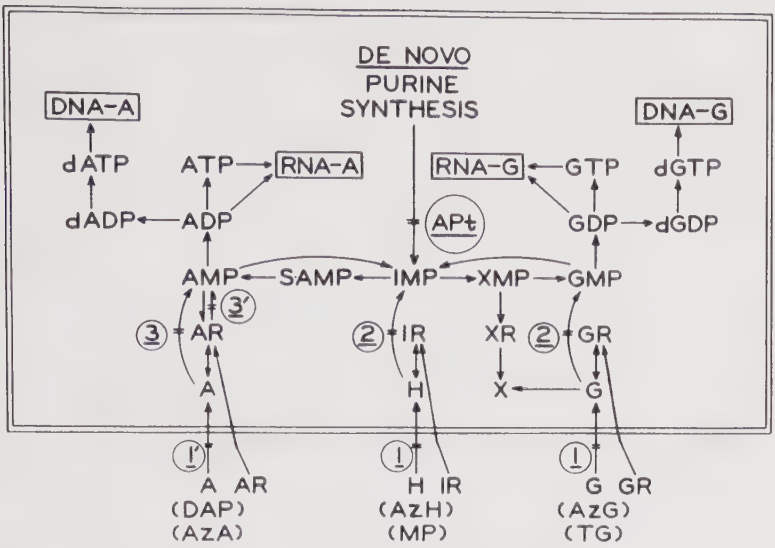


Fig. 9.—Idealized and abbreviated diagrammatic representation of the *de novo* and *preformed* pathways of purine metabolism in D98 mutant cell lines. A, H, X, G: adenine, hypoxanthine, xanthine, guanine; R: ribosides; MP, DP, TP: mono-, di-, triphosphates; IR: inosine; S-AMP: adenylosuccinic acid; d: deoxynucleotides; *1, 1'*: block in uptake of H, G, 8-azaH (AzH), 8-azaG (AzG), 6-thioG (TG), 6-mercaptopurine (MP), A, 8azaA (AzA), 2,6-diaminopurine (DAP), prior to ribophosphorylation; *2*: block in ribophosphorylation of H, G, and their analogues; *3*, 3': partial or complete block in ribophosphorylation of A and its analogues, and in AMP kinase activity, respectively; APt: aminopterin-imposed block of *de novo* pathway of inosinic acid biosynthesis (HAT medium). Mutation from D98S to D98/AG corresponds to blocks *1* and *1'*; from D98/AG to D98/AGR to partial block of *2*; from D98/AG to D98/AH to complete block of *2*; from D98/AH to D98/AH-R to partial restoration of pathway 2. (Reproduced from Szybalski and Szybalska.[33])

3. Selection of Drug-Sensitive Cell Lines.—As apparent in Figure 9, there are two general mechanisms whereby the cell obtains the purine nucleotides needed for nucleic acid synthesis: the *de novo* pathway of inosinic acid (IMP) synthesis, and the *preformed* pathways involving conversion of purine bases to their nucleotides. Since the *de novo* pathway functions perfectly under normal conditions, loss of the *preformed* pathway for hypoxanthine in the D98/AH mutant does not affect its growth on E_{90} medium. However, when the *de novo* pathway is blocked, in this case by adding aminopterin (APt)[17,18] to the medium, and the preformed purine source, hypoxanthine, is supplied, only cells equipped with the IMP-pyrophosphorylase can survive. Thus, E_{90} medium containing APt (0.02 μg./ml.), hypoxanthine (5 μg./ml.), and also thymidine (5 μg./ml.), the latter to overcome the APt-imposed block in the deoxyuridylic-to-thymidylic acid conversion (HAT medium), selects only for cells able to utilize hypoxanthine as sole purine source, e.g. the AH-sensitive lines, D98S or D98/AG, while eliminating D98/AH cells (see Table 1).

This selective system was employed for the isolation of hypoxanthine-utilizing "revertants" from the D98/AH line.[19] Such "revertants," designated

D98/AH-R, occur at a frequency of approximately 10^{-4} in one subline of the D98/AH strain, as shown in Figure 10. When these clones were isolated and characterized, they were found to have regained completely the capacity to utilize hypoxanthine as sole purine source, i.e., when plated at increasing concentrations of the latter in the presence of APt and thymidine a survival curve similar to that exhibited by the wild-type D98S strain was obtained (Figure 10). However, with respect to sensitivity to guanine and hypoxanthine ana-

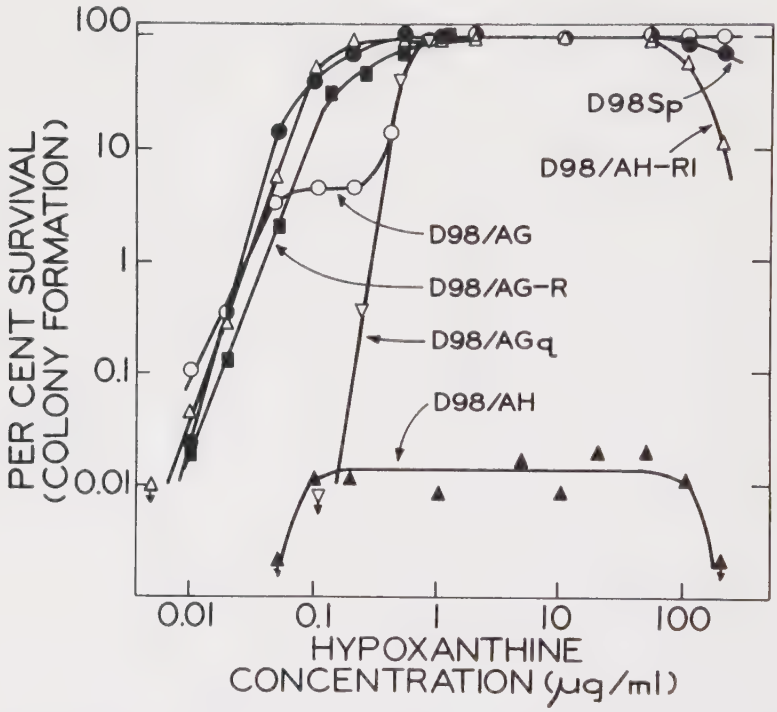


Fig. 10.—Relative capacities of mutant cell lines to utilize hypoxanthine during inhibition of *de novo* purine synthesis by 0.02 μg. aminopterin per ml. in the presence of 10 μg. thymidine per ml. (AT medium), expressed as the ability to form colonies (percent of the control on E_{90} medium). The derivation of the strains is indicated in Figure 4. Strain D98/AGq was obtained by repeated passage of the D98/AG line in AG-containing (6 μg./ml.) E_{90} medium. Strain D98/AH-2, used as a recipient in the transformation experiments (cf. Table 2), could not be included in this figure, since it failed to form any colonies in AT medium even at the highest hypoxanthine level tested.

logues, the D98/AH-R isolates did not appear to be true revertants, i.e., they differed from the D98/AG line in exhibiting intermediate sensitivity patterns characterized by gradual dropping off of the survival curves (Figures 5,6,7,8). Thus, the D98/AH-R mutant has acquired a selective ability for utilization of the natural base and its riboside, regaining only partial sensitivity to their analogues, ranging from a rather low inhibitory threshold for MP to a residual high level resistance to TG (see Table 1). Compatible with these findings

would be the concept of a new IMP-pyrophosphorylase with modified substrate specificity. Assay of the IMP-pyrophosphorylase activity in cell-free preparations, with hypoxanthine and PRPP as substrates, led to the surprising finding (in view of the efficient utilization of hypoxanthine by D98/AH-R) that the D98/AH-R "revertant" actually possesses less than one tenth of the enzymatic activity of D98S and D98/AG cells.[5,16]

Growth of the D98/AH-R line in high concentrations of MP resulted in isolation of the highly AH-resistant line, D98/AH′, practically indistinguishable from the D98/AH mutant (Table 1, Figure 8). Alternating growth in HAT medium and AH- or MP-containing medium would permit the isolation of many sequential mutant lines characterized by gain and loss of IMP-pyrophosphorylase function, respectively, and thus would provide material for genetic study of the fine structure of the IMP-pyrophosphorylase-controlling locus.[19]

Plating of D98/AG cells on the APt-thymidine medium supplemented with a limiting concentration of hypoxanthine (S-HAT medium, described in the next section) led to the isolation of mutant clones (D98/AG-R) which are able to utilize hypoxanthine as efficiently as the D98S strain (Table 1, Figure 10) but exhibit an AG resistance intermediate between that of the D98S and D98/AG strains (Figure 5).

4. Methods for Isolation of "Purified" Mutant Stocks.—The availability of two reciprocal selective systems, one selecting against sensitive and the other against purine analogue-resistant cells provides a means for eliminating pre-existing sensitive or resistant mutants from the corresponding parental population.[5,19] For example, the frequency of D98/AG mutants in the D98S line subcultured in E_{90} medium containing 5 μg. thymidine, 0.2 μg. APt* and 0.15 μg. hypoxanthine per ml. (S-HAT medium) drops precipitously from as high as 10^{-1} to less than 10^{-4}, since as can be ascertained from Figure 10, D98/AG cells require over 1 μg. hypoxanthine per ml. to overcome APt inhibition. The D98S line thus "purified" was denoted D98Sp (Figures 4 and 5).

By subculturing the D98/AG line in HAT medium, the level of pre-existing AH-resistant mutants can be reduced to less than 10^{-6}, the purified line being designated D98/AGp. On the other hand, the D98/AG line seems to have accumulated D98/AG-R mutants, as inferred from the two-step profile of the survival curve in AT medium supplemented with graded concentrations of hypoxanthine (Figure 10). Subculturing the D98/AG line at 10 μg. AG/ml. led to the isolation of the purified line D98/AGq, in which the proportion of D98/AG-R mutants was drastically reduced (Figure 10; curve D98/AGq).

This "purification" of cell stocks is the basis of a greatly simplified procedure for the determination of the spontaneous and induced mutation rates, as discussed in the following sections of this lecture.

* This high concentration of APt is now used to minimize the chance of first-step APt-resistant mutants emerging. With concentrations of 0.02 μg. APt per ml. (20 times the 50 percent inhibitory concentration) in the S-HAT medium employed earlier, an APt-resistant line of D98Sp cells (thirtyfold increase in APt resistance) was unwittingly derived after several successive purifications.

5. Other Drug-Resistant Mutants.—In addition to the purine analogue-resistant mutants listed in Figure 4, we have isolated the D98/AA line resistant to 8-azaadenine and cross-resistant to other adenine analogues including 2,6-diaminopurine, purine, 2-azaadenine, and 2-fluoroadenine. Other mammalian cell lines resistant to purine analogues (including the antibiotic puromycin) were reported by Harris,[14] Harris and Ruddle,[15] Lieberman and Ove,[10] Brockman et al.,[13] Roosa et al.,[11] and Tomizawa and Aronow.[12] Loss of the corresponding pyrophosphorylase was a likely cause of resistance in most of these cases.[13] Resistance to pyrimidine analogues, 5-bromodeoxyuridine (Djordjevic and Szybalski[6]), 5-fluorouridine (Roosa and Herzenberg[20]), and 6-azauridine (Fisher[21]) has also been described. Other resistance markers have included chloramphenicol (Djordjevic and Szybalski[6]), steroids (Grosser and Swim[22]), 2-deoxy-D-glucose (Barban[23]), and folic acid analogues (DeMars and Hooper,[24] Roosa and Herzenberg,[20] Fisher[21]). In the latter case, increase in folic acid reductase, or differences in the transport of APt, were the mechanisms of resistance.[25,26]

This survey includes only *in vitro* developed resistance in mammalian cell lines and does not include the numerous examples of resistance developed *in vivo* for experimental neoplasms.

APPLICATIONS IN GENETIC STUDIES

1. Determination of Spontaneous Mutation Rates.—The selective systems described here permit determination of the somatic mutation rates for mammalian (human) cells. The principle of the Luria-Delbrück variance test[27] was utilized for this purpose by Szybalski[28] and by Lieberman and Ove.[29] The mutation rates determined by this method were found to vary between 5×10^{-4}/cell/generation (D98S → D98/AG)[28] through 10^{-4} (second-step puromycin resistance),[29] 10^{-5} (first-step aminopterin resistance),[30] 4×10^{-6} (first-step puromycin resistance),[29] to less than 2×10^{-7}/cell/generation (D98/AH-2 → D98/AH-R).[5]

A simplification of the method for determination of spontaneous mutation rates[19] is based on the procedure for removal of pre-existing mutants described above. For example, prior purification of the D98S line in S-HAT medium permits the direct estimation of the mutation rate to AG resistance from the frequency of mutants forming colonies in AG-containing selective medium. Similarly, HAT medium can be used for purification of the D98/AG line prior to determination of the frequency of AH-resistant mutants. A more detailed description of this method was recently summarized elsewhere.[5]

2. Mutagenicity Studies.—Three selective systems were explored for testing the mutagenic effects of several chemical and physical agents. These systems included the mutations from D98Sp, to D98/AG, from D98/AG to D98/AH, from D98/AH to D98/AH-R. In addition, the D98/AG to D98/AG-R (Figure 4) mutational system was utilized to a more limited degree. As first observed with ultraviolet light[31] and as summarized recently,[5] no mutagenic effect was detected for any of the agents tested, including ultraviolet light,

X-ray, nitrogen mustards, triethylene melamine, beta-propiolactone, halogenated thymidine analogues, and others. Moreover, in about half of the cases the spontaneous mutation rates were actually found to be depressed by a factor of 2 to 10. The latter phenomenon could not be accounted for by selective toxicity of the mutagens for the mutants, as ascertained from reconstruction experiments. This problem is at present under active study.

3. Design of Chemotherapeutic Approaches.—*In vitro* tissue culture studies could serve as models for designing means to circumvent the omnipresent problem of resistance development during antineoplastic therapy. Knowledge of the mutation rates toward drug resistance and of the biochemical mechanisms of resistance in human cell lines *in vitro* is a basic prerequisite to planning the strategy of the rational chemotherapeutic approach. For example, in the case of clinical resistance to purine analogues, the question arises whether the resistance is of the D98/AG type* or whether the loss of a pyrophosphorylase is involved (the D98/AH type). Depending on the occurrence of one or another of these types of resistance, a different therapeutic stratagem should be planned. A scheme for converting the latter type of resistance into sensitivity was proposed by Szybalski and Szybalska,[33] and other similar approaches, including the phenomenon of "collateral sensitivity" (Szybalski and Bryson[7]), could be devised. There is little doubt that human cell line models approximate more closely clinical neoplasms than the bacterial systems widely employed in drug resistance studies.

4. Genetic Interactions.—Drug resistance markers have not been applied in quantitative fashion for the detection of genetic recombination between intact mammalian cells. The only published studies on the use of morphological chromosomal markers for the detection of cell recombinants[34–36] suffer from inadequate quantitation, a prerequisite for meaningful genetic analysis.

Taking advantage of the good selective system available for the isolation of IMP-pyrophosphorylase-producing cells from the D98/AH population (HAT medium), experiments designed to detect DNA-mediated genetic transformation of that character were undertaken. The D98S or D98/AG line served as the DNA donor, and D98/AH-2 (a subline of the D98/AH strain with no measurable spontaneous "reversion" rate) was the recipient. The conditions of the transformation experiments were described earlier[5,37] and are summarized in Table 2. Recipient cells were exposed to donor DNA in concentrations ranging from 0.5 to 32 μg./ml. for periods up to 30 minutes, in a medium composed of balanced saline with 4 percent glycerol and 50 μg./ml. spermine, while either in suspension or attached to glass. Under these conditions there was no loss of colony-forming cells during the period of treatment. The cells were then plated in HAT medium and the number of colonies scored after two weeks' incubation.

* Some "wild-type" neoplasms could already be endowed with the D98/AG type of resistance. For example, the H.Ep.#1 line derived from a human neoplasm is 100 times more resistant to AG than to AGR and thus closely resembles the D98/AG mutant.[32]

TABLE 2.—*DNA-Mediated Genetic Transformation in D98 Cells: Transfer of Capacity to Utilize Hypoxanthine from D98/AG Donor to D98/AH-2 Recipient Cells*

DNA Source	DNA Concentration (μg./ml.)	No. of Colonies on HAT Medium* per 3.75×10^5 D98/AH-2† Recipient Cells	Transformant Frequency
None	0	0	$< 2 \times 10^{-7}$‡
D98/AG†	0.5	1	0.3×10^{-5}
D98/AG†	2	7	1.9×10^{-5}
D98/AG†	8	10	2.7×10^{-5}
D98/AG†	32	45	12.0×10^{-5}
D98/AG†	32 (DNase-treated)	0	$< 0.3 \times 10^{-5}$
D98/AH-2†	8	0	$< 6 \times 10^{-7}$‡
D98/AH-2†	32	0	$< 6 \times 10^{-7}$‡

* E_{20} medium containing aminopterin (0.02 μg./ml.) + thymidine (5 μg./ml.) + hypoxanthine (5 μg./ml.).

† The IMP-pyrophosphorylase-deficient (IMP-PPase$^-$) D98/AH-2 line served as the recipient. Donor DNA was isolated from sodium lauryl sulfate-lysed, IMP-PPase$^+$ D98/AG cells (and from D98/AH-2 cells as a control) and purified either by phenol deproteinization in conjunction with RNase treatment or by preparative CsCl-gradient centrifugation.

‡ Based on pooled control data from several experiments. No colonies were ever formed on HAT medium by D98/AH-2 cells untreated or exposed to D98/AH DNA.

In a series of transformation experiments employing this system, the following results were obtained. Colonies were formed on HAT medium only by D98/AH-2 cells which had been treated with DNA isolated from IMP-pyrophosphorylase-producing (D98S or D98/AG) donors, in the presence of spermine. No colonies at all were ever observed in control plates seeded with equal inocula of recipient cells which were untreated or exposed to (a) DNA extracted from the recipient D98/AH cells, (b) DNase-digested D98/AG DNA, or (c) bacteriophage T6 DNA. The frequency of "transformant" colonies formed by donor DNA-treated cells ranged from 3×10^{-6} to 10^{-4}, increasing with the DNA concentration (Table 2). When several of these clones were isolated, they were found to breed true and to be indistinguishable from the DNA donor parent with respect to their ability to utilize hypoxanthine and their sensitivity to guanine and hypoxanthine analogues. It was concluded[5] that "the phenomenon observed has many features in common with, or is indeed identical to, the DNA-mediated transformation observed in bacteria."

It is important to stress that the term "transformation" has been used in a very loose sense in most of the literature pertaining to tissue culture studies and has been applied to (a) uncontrolled heritable changes in the growth pattern of fresh cell explants,[38] probably a major chromosomal mutation or other rearrangement, (b) similar changes caused by infection with a virus or viral nucleic acid,[38] and (c) temporary changes in synthetic pattern elicited by the extracellular addition of nucleic acids.[39] The term "DNA-mediated genetic transformation" is used here with the same meaning implied as in bacterial

genetics, i.e. a heritable change in the well controlled genetic characteristics of the recipient cell produced by highly polymerized DNA extracted from cells bearing the new character.

The reciprocal approach, i.e. the attempt to transform cells toward increased purine analogue resistance, was considered less promising because of the likelihood of a greater phenotypic lag in the expression of a genetic change in the direction of decreased enzyme activity. Nevertheless, preliminary experiments have been carried out, employing the clear-cut selective systems available for the isolation of D98/AG and D98/AH mutants from sensitive populations. Under the conditions employed, inadequate resolution was obtained with these systems, due to either too high a spontaneous mutation rate or a very low transformation rate.*

CONCLUSIONS AND SUMMARY

Reciprocal selective systems permitting the isolation and the scoring of human cell mutants have been developed: (1) a system selecting toward purine analogue resistance, associated with partial impairment or loss of the metabolic pathway for utilization of preformed purines or their analogues, and (2) the reverse system selecting toward regain of the latter pathway, associated with increased capacity to convert the purine bases to their nucleotides (IMP-pyrophosphorylase activity). Several useful markers were developed: increased resistance to 8-azaguanine (D98/AG), 8-azaguanosine (D98/AGR), and 8-azahypoxanthine (D98/AH) (selective systems: type 1); and regained capacity to utilize limiting concentrations of hypoxanthine under conditions of selective inhibition of *de novo* inosinic acid synthesis by aminopterin (D98/AH-R, D98/AG-R) (selective systems: type 2). Since the latter mutations do not restore completely the wild phenotype, genetically they may belong to the class of suppressor mutations. The biochemical basis for the mutant phenotypes was partially elucidated and the selective systems were applied in a variety of genetic experiments, including determination of the spontaneous mutation rates, evaluation of chemical and physical mutagens, and the demonstration of DNA-mediated genetic transformation in human cells. A more detailed account of our studies pertaining to DNA-mediated genetic transformation in human cell lines will be published elsewhere.[40]

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* An example of failure to transform mammalian cells toward drug resistance (amethopterin) was reported after this manuscript was completed (Mathias and Fisher[30]). One could rationalize about whether this result is inherent in the cell line or mutational system employed, or reflects a transformant frequency lower than the frequency of spontaneous mutants (10^{-5}) especially since spermine, obligatory in our experience, was not used. The rather involved method of DNA preparation employed might have imparted more shear to the DNA than our simplified procedure involving only lysis (phenol or sodium lauryl sulfate) of the cells in the presence of Mg^{++} chelators (sodium citrate or Versene) followed either by gentle phenol deproteinization in conjunction with RNase treatment or by the CsCl gradient centrifugation procedure.[6]

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REFERENCES

1. Ryan, F. J.: Applicability of Microbial Genetics to Characterization of Mammalian Cells. Univ. Michigan M. Bull. *28*:267–276, 1962.
2. Puck, T. T.; Marcus, P. I.; and Cieciura, S. J.: Clonal Growth of Mammalian Cells *in vitro*. J. Exp. M. *103*:273–283, 1956.
3. Berman, L., and Stulberg, C. S.: Eight Culture Strains (Detroit) of Human Epithelial-Like Cells. Proc. Soc. Exp. Biol. *92*:730–735, 1956.
4. Szybalski, W., and Smith, M. J.: Genetics of Human Cell Lines; I. 8-Azaguanine Resistance, a Selective "Single-Step" Marker. Proc. Soc. Exp. Biol. *101*:662–666, 1959.
5. Szybalski, W.; Szybalska, E. H.; and Ragni, G.: Genetic Studies with Human Cell Lines. National Cancer Institute Monograph 7, 1962, pp. 75–88.
6. Djordjevic, B., and Szybalski, W.: Genetics of Human Cell Lines; III. Incorporation of 5-Bromo- and 5-Iododeoxyuridine into the Deoxyribonucleic Acid of Human Cells and Its Effect on Radiation Sensitivity. J. Exp. M. *112*:509–531, 1960.
7. Szybalski, W., and Bryson, V.: Genetic Studies on Microbial Cross Resistance to Toxic Agents; I. Cross Resistance of *Escherichia coli* to Fifteen Antibiotics. J. Bact. *64*:489–499, 1952.
8. Szybalski, W., and Bryson, V.: Origin of Drug Resistance in Microorganisms. In Sevag, M. G.; Reid, R. D.; and Reynolds, O. E. (Eds.): *Origin of Resistance to Toxic Agents*. New York: Academic Press, Inc., 1955, pp. 20–41.
9. Szybalski. W.: Resistance to 8-Azaguanine, a Selective Genetic Marker for a Human Cell Line. Microb. Genetics Bull. *16*:30, 1958.
10. Lieberman, I., and Ove, P.: Isolation and Study of Mutants from Mammalian Cells in Culture. Proc. Nat. Acad. Sc. *45*:867–872, 1959.
11. Roosa, R. A.; Brockman, R. W.; and Law, L. W.: Resistance to 8-Azaguanine (AZG) in P388, a Lymphocytic Leukemia in Cell Culture. Proc. Am. Assn. Cancer Res. *3*:262, 1961.
12. Tomizawa, S., and Aronow, L.: Studies on Drug Resistance in Mammalian Cells. II. 6-Mercaptopurine Resistance in Mouse Fibroblasts. J. Pharm. Exp. Ther. *128*:107–114, 1960.
13. Brockman, R. W.; Kelley, G. G.; Stutts, P.; and Copeland, V.: Biochemical Aspects of Resistance to 6-Mercaptopurine in Human Epidermoid Carcinoma Cells in Culture. Nature *191*:469–471, 1961.
14. Harris, M.: Specificity and Cross-Reactions in a Strain of Pig Kidney Cells Resistant to 2,6-Diaminopurine. Exp. Cell Res. *21*:439–446, 1960.
15. Harris, M., and Ruddle, F. H.: Growth and Chromosome Studies on Drug Resistant Lines of Cells in Tissue Culture. In *Cell Physiology of Neoplasia*. Austin: University of Texas, 1960, pp. 524–546.

16. Szybalski, W.; Szybalska, E. H.; and Brockman, R. W.: Biochemical Basis of Sequential Mutations Toward Resistance to Purine Analogs in Human Cell Lines. Proc. Am. Assn. Cancer Res. *3*:272, 1961.
17. Werkheiser, W. C.: Specific Binding of 4-Amino Folic Acid Analogues by Folic Acid Reductase. J. Biol. Chem. *236*:888–893, 1961.
18. Hakala, M. T.: Prevention of Toxicity of Amethopterin for Sarcoma-180 Cells in Tissue Culture. Science *126*:255, 1957.
19. Szybalska, E. H., and Szybalski, W.: Selective Systems for Measuring Forward and Reverse Mutation Rates Involving Loss and Gain of an Enzyme in Human Cell Lines. Abstracts of Communications, V Internat. Cong. Biochem. Moscow: Pergamon Press, Ltd., 1961, p. 411.
20. Roosa, R. A., and Herzenberg, L. A.: The Selection of Variants Resistant to Folic Acid Antagonists and 5-Fluorouridine in Cell Cultures of a Lymphocytic Neoplasm. Proc. Am. Assn. Cancer Res. *3*:58, 1959.
21. Fisher, G. A.: Nutritional and Amethopterin-Resistant Characteristics of Leukemia Clones. Cancer Res. *19*:372–376, 1959.
22. Grosser, B. I., and Swim, H. E.: *In Vitro* Response of Cultured Uterine Fibroblasts to Various Steroids. Proc. Am. Assn. Cancer Res. *2*:304, 1958.
23. Barban, S.: Studies on the Mechanism of Resistance to 2-Deoxy-D-glucose in Mammalian Cell Cultures. J. Biol. Chem. *237*:291–295, 1962.
24. DeMars, R., and Hooper, J. L.: A Method for Selecting for Auxotrophic Mutants of HeLa Cells. J. Exp. M. *111*:559–572, 1960.
25. Hakala, M. T.; Zakrzewski, S. F.; and Nichol, C. A.: Mechanism of Resistance to Amethopterin in Sarcoma 180 (S-180) Cells in Culture. Proc. Am. Assn. Cancer Res. *3*:115, 1960.
26. Fisher, G. A.: Increased Levels of Folic Acid Reductase as a Mechanism of Resistance to Amethopterin in Leukemic Cells. Biochem. Pharmacol. *7*:75–77, 1961, and personal communication.
27. Luria, S. E., and Delbrück, M.: Mutations of Bacteria from Virus Sensitivity to Virus Resistance. Genetics *28*:491–511, 1943.
28. Szybalski, W.: Genetics of Human Cell Lines. II. Method for Determination of Mutation Rates to Drug Resistance. Exp. Cell Res. *18*:588–591, 1959.
29. Lieberman, I., and Ove, P.: Estimation of Mutation Rates with Mammalian Cells in Culture. Proc. Nat. Acad. Sc. *45*:872–877, 1959.
30. Mathias, A. P., and Fisher, G. A.: Transformation Experiments with Murine Lymphoblastic Cells (LS178Y) Grown in Culture. Biochem. Pharmacol. *11*:69–78, 1962.
31. Szybalski, W., and Smith, M. J.: Effect of Ultraviolet Light on Mutation to 8-Azaguanine Resistance in a Human Cell Line. Fed. Proc. *18*:336, 1959.
32. Rich, M. A.; Perez, A. G.; and Eidinoff, M. L.: The Effects of Some Purine Analogs on the Growth of H. Ep. #1 and Chick Embryo Fibroblast Cells. Cancer Res. *22*:3, 1962.
33. Szybalski, W., and Szybalska, E. H.: A New Chemotherapeutic Principle for the Treatment of Drug-resistant Neoplasms. Cancer Chemother. Rep. *11*:87–89, 1961.
34. Barski, G.; Sorieul, S.; and Cornefert, F.: "Hybrid" Type Cells in Combined Cultures of Two Different Mammalian Cell Strains. J. Nat. Cancer Inst. *26*:1269–1291, 1961.
35. Sorieul, S., and Ephrussi, B.: Karyological Demonstration of Hybridization of Mammalian Cells *In Vitro*. Nature *190*:653–654, 1961.
36. Barski, G.: Clones Cellulaires "Hybrids," Isolés á Partir de Cultures Cellulaires Mixtes. C. rend. Acad. sc. *253*:1186–1188, 1961.
37. Szybalski, W., and Joannes, S. A.: Genetic Transformation in Human Cells. Microb. Genetics Bull. *18*:22–23, 1962.
38. Swim, H. E.: Microbiological Aspects of Tissue Culture. Annual Rev. Microb. *13*:141–176, 1959.
39. Kraus, L. M.: Formation of Different Hemoglobins in Tissue Culture of Human Bone Marrow Treated with Human Deoxyribonucleic Acid. Nature *192*:1055–1057, 1961.
40. Szybalska, E. H., and Szybalski, W.: Genetics of Human Cell Lines. IV. DNA-Mediated Heritable Transformation of a Biochemical Trait. Proc. Nat. Acad. Sc. (in press).

Chromosome Morphology as a Genetic Marker

Paul S. Moorhead, Ph.D.

THE correlations between the cytologically visible chromosome and the phenotypic expression constitute the "stock in trade" of the cytogeneticist. Although cytogenetics is a morphological and descriptive science, it deals with a very special morphology, that of the genetic content itself.

The early work of Curt Stern[110] in which he proved the cytological basis of crossing over stands as a classic model for the field. Stern obtained a Drosophila stock heterozygous for both carnation (a recessive) and Bar (a dominant) carried on morphologically abnormal X chromosomes. These particular X chromosomes were formed by translocations, one being an X with chromosomal material from the Y carried as a short arm and the other having a generally reduced length since much of its material was translocated to chromosome IV. Upon mating with a normal male heterozygous for carnation, the crossover classes among females would be either Bar or carnation in phenotype. However, the visible morphology of the X's in these crossover classes was like neither of the two maternal marked X's since the crossover exchange had altered their visible morphology in a predictable fashion.

It seems an anachronism that in these days of "molecular biology" our interests and attention should be directed to microscopic studies of chromosome morphology. However, chromosomes are having their own renaissance and much of the more recent interest centers upon the possibility of finding the means for some form of "infective heredity" in the cells of laboratory animals and in man himself.

The basic descriptive information of the karyotype of man and of many mammals has only recently been made available to us,[2,27,64,116,117] and the exploitation of these newly developed facts is not yet complete. As recently as 1957 and 1958 most publications concerned with the chromosome numbers of mammalian cell lines *in vitro* referred to "counts" as correct to "plus or minus two," whereas currently counts are "exact" except for occasional artifacts due to over-spreading. These useful advances in techniques for the preparation of mitotic cells have grown out of the use of ascites tumor cell populations[60,66,68,115] and afterward through the widespread use of tissue-cultured mammalian cells.[28,54,95,118]

The present paper is a review of some selected works bearing on the use of chromosome markers in animal cells *in vitro* as related to the possibilities of the development of "Somatic Cell Genetics." Insofar as the cytologic localiza-

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tion of genetic factors or the mapping of mammalian chromosomes is concerned, very little exists that can properly be termed a part of somatic cell genetics; however, a number of interesting leads and possibilities have been uncovered. For this reason this presentation is necessarily exploratory and speculative. For broader considerations of this subject the reader is referred to recent reviews by Lederberg,[62] Allen,[1] Schultz,[107] Hsu,*[48] and Klein.*[58]

KARYOMERE MARKERS OF NUCLEAR INTERPHASE

Before the consideration of chromosomes proper, it may be of interest to mention some cell markers of great potential utility; that is, interphase markers, which do not require elaborate techniques.

Upon completion of anaphase each group of chromosomes will form a restitution nucleus and certain gross characteristics of the group may still be discernible in the interphase nucleus. In one of Ruddle's porcine cell lines,[96] there was noted an unusually long marker chromosome which protrudes toward the cleavage plane so that such nuclei are conveniently recognizable in the interphase state. A similar characteristic protrusion of several chromosome ends in the restitution nucleus of Ascaris was cited by Boveri[11] as evidence for the retention of chromosome individuality during the functional state between successive divisions.

The degree of polyploidy can be estimated crudely from nuclear size, at least in material which is essentially diploid, tetraploid, etc., but lacking in heteroploid types.

A classic example of the determination of the ploidy state of interphase nuclei was that of Geitler[34] in which he enumerated the prochromosomes or heteropyknotic bodies in the interphase nuclei of high ploidy in the Pond Skater. More recently, the use of sex chromatin or the Barr body as an indicator of the number of X chromosomes has become an extensive field in itself with important ramifications.[5,43,59]

Although much of the work with the Barr body employs Feulgen or special staining techniques to heighten the differentiation, orcein serves well or even Giemsa staining will permit a ready distinction between monolayer cultures of male and female diploid human cells.[44] A variety of hypotheses exists as to the nature of sex chromatin.[5]

A proposed formula which is reasonably consistent with a variety of abnormal sex chromosome conditions in man has been offered by Harnden[38] which is $B = X - p/2$.† In cultures of XXXY-48 human tissue, a few very large nuclei are seen to possess 8 Barr bodies and if these are octoploid as Harnden presumes, they would clearly fit the hypothesis. Tetraploid nuclei, which occur at 2 to 3 percent frequency, display 2 Barr bodies in normal fe-

* Grateful acknowledgment is made to T. C. Hsu and G. Klein for making their manuscripts available prior to publication.

† Number of Barr bodies per nucleus is equal to number of X chromosomes minus one-half the ploidy level.

male material and of course diploid nuclei show only one. Tetraploid and diploid male (XY or XO) nuclei show no sex chromatin in general.

General histologic differences in nuclear morphology between species were ably exploited as markers by Moscona[78] in his work in which he demonstrated that *in vitro* chimeric cell reaggregation occurred on the basis of type [organ] of cell rather than on the recognition of species. Mouse nuclei were easily distinguished from chick nuclei in tissue-cultured mixtures and even in fusion cells containing both species of nuclei.

Another example of the use of morphological patterns seen in interphase is illustrated in the work of Chu and Giles[18] who have undertaken studies to determine the morphological significance of the patterns of nucleoli by detailed analysis of their form, volume, and numbers in diploid human material *in vitro*.

In this connection the interesting studies on wheat by Longwell and Svihla[71] have shown that the function associated with the formation of nucleoli can be assumed by other latent chromosomes in the absence of the principal nucleolar-organizing chromosome. These authors studied a series of monosomic races, each monosomic for a particular chromosome. They found that when the principal nucleolar chromosome was tetrasomic, there resulted a 50 percent increase in cytoplasmic volume. Such obvious effects should have experimentally practical possibilities for the RNA biochemist if a parallel could be demonstrated in a mammalian cell.

GENERAL CONSIDERATIONS OF CHROMOSOME FORM

The metaphase chromosome has evolved as the cell's solution to the problem of achieving an equal and orderly distribution of the original genetic code and its replicated copy to the two daughter cells. Presumably with evolution toward greater complexity, the greater length of the chromosome could limit the increase in loci for simple mechanical reasons. The typical chromosome at metaphase is then a "package" of the linearly arranged gene factors and is manageable through the mitotic process only by virtue of a process of coiling. The cytologically visible coils occur at various levels but do not have any directly evident basis in the postulated molecular DNA helix. Also evolved was a "handle" for the "package" in the form of the kinetochore or centromere, the attachment point for the spindle fiber. Enumeration of these centromeres then determines the "chromosome number" of a cell or cell population since acentric fragments do not persist except by occasional passive inclusion within a restitution nucleus.

The process of condensation by which the chromosomes first become visible at prophase proceeds to its maximum at anaphase and the resulting reduction in apparent length of all the chromosomes is quite large. The usual employment of colchicine for observations on somatic metaphases adds a further complication by affecting chromosome coiling as well as the spindle. Whether chromosomes of a dividing cell are spread by squashing or by air-drying techniques, the possibility of mechanical forces causing stretching artifacts of these coiled structures is always present. In spite of these considerations, the length of a chro-

mosome is still a primary datum in the differential recognition of metaphase chromosomes. The position of the primary constriction or centromere is the most valuable parameter available to us, involving as it does the *relative* lengths of the parts of a single chromosome. In comparisons within one chromosome, the two arms must have some factors in common affecting variability of coiled length. In air-dried metaphases from normal peripheral blood leukocyte cultures the clearly identifiable homologues of human chromosome #1 will occasionally show as much as a 20 percent difference in length. The difference in the centromeric position or arm length ratio of human chromosome #1 (1.1.) as against that of #2 (1.5) is, however, quite sufficient to permit an easy distinction. Thus, arm ratio is considerably more reliable than over-all chromosome length although the error becomes large for high arm ratios. Emphasizing the distortions of size that are unavoidably introduced, Leighty and Plaisted[63] have statistically analyzed the sources of variance in respect to length and to the mean ratio of the arms of three chromosomes in Lilium. They point out that such biological hierarchies as source of clone, plant, and root tip contribute very little to the total variance as compared to those hierarchies which become involved following cytological manipulations such as slide, cell, or homologue.

Other "taxonomic" features available in chromosome recognition are the positions of secondary constrictions and satellites and also possibly heteropyknosis of the sex chromosomes.[80] However, differences in density of staining are sporadically seen in various autosomes, too.

In the derivation of a cell karyotype, which is a formalized version of the chromosome complement expressed in relative and absolute lengths with other observed detail noted, a number of high quality metaphase configurations must be chosen. In this sense the chromosome cytologist "selects his data" but in a way to minimize the variables, and his skill in this can improve with increasing familiarity with his material. The possibilities for misleading himself and others are sometimes great[83] and acceptable proof that the features of a chromosome truly reflect a consistent or innate morphological difference is difficult in the absence of some clear advantage such as exists in the giant polytenic chromosomes of Drosophila.

For these reasons the choice of a marker chromosome in any experimental design is usually made from among aberrant chromosomes created by gross structural rearrangements such as reciprocal translocations between nonhomologous chromosomes. Ideally, it is desirable that immediate and unequivocal recognition in each cell examined be possible in contrast to a general or statistical differentiation as between populations. The latter distinction, as between HeLa parental type and HeLa S1 or S3, can be made on the basis of a frequency distribution of chromosome centromeric types: metacentric, telocentric and submetacentric.[17,24]

An inversion of a segment of a chromosome with its two break points to either side of a centromere and necessarily *not* equidistant from it will create a marker chromosome with a recognizably different arm ratio. Such a marker is

theoretically preferable to that formed by a deletion or duplication of a part or of a whole chromosome since less violence in the way of genetic disturbance may be presumed in an inversion of gene order than in the changes in gene dosage effected by duplication-deficiency types of rearrangements. In an inversion or even in a Robertsonian ("whole-arm") translocation,[81] essentially all the genetic material is retained within the karyotype. Theoretically even the mildest rearrangement could induce some sort of position effect but this risk is negligible as compared to genetic duplications and deficiencies, especially of the order that would be required for the changes to be cytologically visible. The abnormally reduced length of the Ph_1 chromosome found in some of the leucocytes of chronic granulocytic leukemia patients[79] represents perhaps the limit to a detectable difference between a new or "marker" chromosome and the normal. This detection of the absence of less than one half of the longer arm of one of the smallest chromosomes in man certainly could not have been made if the missing part were deleted (or translocated) from one of the larger chromosomes of the 6-12 group.

Duplication of an entire chromosome by a nondisjunctive type of mitotic error should be regarded as rather drastic in respect to the possible phenotypic effects. Down's syndrome and Klinefelter's syndrome are phenotypic examples of such trisomy. Whether chromosome changes are involved in the origin of neoplasia or not,[7] their implication in tumor progression[32] should serve to warn us off concerning the use of changes at the level of trisomy.[20,29,40] Mouse neoplasias frequently are seen to possess chromosome changes of one or two extra chromosomes.[112] In the latter work 15 of 16 induced malignancies were found to have only one extra chromosome and the remaining case had a complement of 42 chromosomes ($2n + 2$).

Thus far in instances involving the use of marker chromosomes *in vitro*, either the material itself was grossly heteroploid or genetically unknown, or both, so that consideration of any related phenotypic effects seems premature. Klein[58] has emphasized that a system for studies of somatic cell genetics should be based upon genetically defined material with cells from animals which have been inbred. The extensive and growing genetic and immunologic knowledge of the mouse[25] would make this a choice mammalian material except for the discouraging similarity between the 20 pairs of chromosomes of its karyotype.[56] Griffen's work on the mapping of the mouse pachytene chromosomes may offer some encouragement as do the increasing number of findings concerning certain aneuploid conditions in mice.[36,99,100]

Even though the Y chromosome of the mouse has been described in somatic mitosis,[113] its identification hinges on a slight size difference and is hardly ideal from the standpoint of clean cell-by-cell separations. There are also one or two pairs having a strong secondary constriction but the conditions affecting the variable expression of secondary constrictions seen at metaphase are not yet understood.

Hsu and Somers[55] have reported on the exaggeration of secondary constrictions in mammalian cells in culture resulting from the presence of an abnor-

mal metabolite, 5-bromodeoxyuridine. Similar striking exaggerations were seen in chromosomes #1, #9, and #16 in human diploid cells exposed to tritiated thymidine for studies of possible asynchrony of uptake.[76] However, subsequent study has shown that this enhancing effect on the secondary constrictions was due to the cytological technique used for spreading, which is to ignite the fixative to hasten evaporative drying on the slide.

As Yerganian[125] has reiterated, the Chinese hamster offers exceptional possibilities for those interested in experimental cytogenetics. Yerganian[31,126] has performed a number of interesting studies concerning the karyology and stability of Chinese hamster cells *in vitro*. The superiority of a cell with only 11 easily distinguished chromosome pairs is clearly pointed up by his findings of diploid counts which are actually "quasi-diploid." Although the total count is 22, quasi-diploids do not possess 2 complete haploid sets but may be simultaneously trisomic and monosomic *or* be tetrasomic and nullsomic for different specific chromosomes.

Work with chromosome markers in mammalian cells has so far consisted almost entirely of using the marker as a phenotype, that is, using it to obtain evidence of clonal continuity or cell lineage without regard to the particular genetic content of the marker itself.

The work of Ford and his co-workers[4,29,30,75] with the T6 mouse chromosome marker may be taken as representative of experiments using the abnormal chromosome as an identification tag, in this case for studies concerning the repopulation of an irradiated host with inoculated T6 cells. The T6 chromosome is very small, the result of a reciprocal translocation. Using a heterozygous T6/+ mouse stock obtained by Carter and collaborators,[15] Ford and co-workers[29] were able to conclude that the clonal progeny of a single chromosomal mutant from the host's cells could successfully repopulate various hemopoietic sites. This could be stated conclusively since a novel long chromosome spontaneously arose during the experiment and was recognizable in the spleen, bone marrow, and in two lymph nodes of one animal. In the course of finding a suitable marker at the beginning of their work, Ford states that the T6 was the only satisfactory aberrant chromosome from among 22 arising from 11 reciprocal translocations. This re-emphasizes the poverty of the mouse karyotype for our purposes.

SEX CHROMOSOME HETEROMORPHY

The use of the naturally heteromorphic sex chromosomes as markers sidesteps the problems of possible extraneous genetic influences introduced by the marker itself. As to the genetic content of X and Y, those differences normally associated with sex may of course be taken into account, at least theoretically.

An interesting example of the use of sex chromosome morphology for the detection of a mixed population, a "mosaique artificielle," was given in the work of Lejeune and Turpin.[64] Skin was grafted from one of a pair of presumed monozygotic twins to the other. The donor twin had a chromosome constitution of XY and the other of XO, and after 77 days the successful graft

was biopsied and cultured for determination of the karyotypes present. Dividing cells of both XY and XO types were obtained, thus supporting the view that these were monozygotic twins and that the original aberrant division occurred after formation of the zygote, possibly at the first division of the zygote. These considerations have been exploited by others[124] in mosaic human material in which XX versus XY (or XO) is readily determined even though the X itself is difficult to identify individually.

A similar use of the sex chromosomes as markers, but in an *in vitro* situation, was made in the identification of the surviving cell strain in an experiment concerning the cause of the characteristic demise of long term cultures of human diploid cell strains.[44] After several months (45 to 55 passages) *in vitro,* human fibroblast diploid cell strains invariably but unexplainedly undergo a loss of proliferative vigor and eventually degenerate. To rule out possible gross infection or deficiences of the medium as the cause of this, a vigorous early-passage diploid cell strain which was female was seeded into a male strain culture which was in its degenerative phase (49th passage). The recovery, after 17 further subcultivations, of exclusively XX cells shown by chromosome study, indicated that the "younger" strain could easily overgrow the failing male strain although sharing the same *in vitro* environment. The inoculated female strain was itself spent after 40 to 50 *total* subcultivations as was a sister subline unexposed to the male strain.

J. Ponten[85] has recently been able to show in a series of studies that the supposed transplantation of erythroblastosis viral leukemia via cells injected into a new chicken host may in fact not be a transplantation at all but may represent a fresh induction of the neoplasia in the host cells. The distinction between male host and female donor cells, as well as in the opposite arrangement, was clearly evident by noting the presence of one Z (or X) chromosome in female cells and two Z's in male cells. The sex chromosomes of the chicken are large enough to be observed easily and are unambiguous in arm ratio. If the cellular composition of the bone marrow and spleen was followed during several transplant generations, the original donor cells failed to dominate and were gradually replaced by host cells induced to proliferate in the recipients. Burmester's line of inbred chickens was used to insure optimal conditions for survival of the inoculum into the host. Similarly, Ponten demonstrated that inoculated cells are eventually eliminated from the host in studies on Rous sarcoma, indicating that viral induction is the principal mechanism of its transfer and not transplantation. Another virus-associated neoplasm, the RPL-12 lymphoid tumor, was, on the other hand, found to be homologously transmitted by true transplantation.

For the kind of markers considered above, the tagging of cell lineage, the heteromorphic sex chromosomes are perhaps ideal, but we have barely begun to work with cells of normal karyotype which have a definite unit character recognizable *in vitro*. The *in vitro* demonstration by Krooth and Weinberg[61] of the galactosemic condition is a notable beginning in this direction.

For *in vitro* systems, a stable karyotypic background, as is available in

human "fibroblastic" diploid strains, is almost a *sine qua non*.[19,44,87] Before we consider some of the problems concerning the stability of the chromosomes of diploid cells in culture, some of the experience with heteroploid cell lines should be reviewed.

HETEROPLOID CELL LINES

Following the introduction to tissue culture experimentation of the human cell line HeLa by Gey[35] and of the mouse L cell line by Earle,[26] numerous other lines were obtained in various laboratories, and interest in viral and immunologic capabilities led to a number of reports describing the process of *in vitro* "alteration" or "transformation."[8,74,121] It was soon determined that all the cell lines so examined possessed a grossly abnormal chromosomal complement,[53,67,98] whether the line had originated from malignant or normal tissue. Differences of opinion about whether the changes observed were wholly genetic or represented forms of modulation of the cell were common. In fact, considering the confusion of terminology regarding morphological changes *in vitro* and lack of chromosome data in many reports, both kinds of events were undoubtedly spoken of under the single heading of "cell transformation." That some of these "transformations" involved antigenic loss or gain of a high order,[23] as mouse versus man, led some to suspect that cell contamination was involved. The first study of this possibility was that of Rothfels et al.[93] in which karyologic criteria were used to unravel some of the confusion. Convincing evidence concerning the centromeric types of chromosomes expected according to species as well as certain very distinctive abnormal chromosomes from the L cell provided convincing grounds for the conclusion that either cell contamination or mislabeling of cultures was responsible for the supposed conversions of many cell lines. These same markers in the L cell were identifiable in sublines of L cells from widely distributed laboratories[51] and were noted in similar comparative studies by others.[13,21,24] In these latter studies immunologic tests (second set reactions and hemagglutination tests) were performed simultaneously with the karyologic studies and provided complete correspondence with regard to the detected contaminations. On one occasion a line received by our laboratory for karyologic confirmation of its presumed chicken origin was found to consist of a mixture of murine and human cells but the murine cells were not L cells since all the chromosomes were telocentric.

The finding of cell contaminations did not, of course, "explain away" all cases of *in vitro* cell alterations, for many were found free of both karyologic and immunologic "accusations." While interspecific differences can be detected, proof of possible contaminations within a species are not usually possible. Many cell lines known to be human may actually be HeLa contaminations; various mouse cell lines are easily distinguished from the better marked L cell, the most common murine line. The derivation of many cell lines proceeded rapidly[16] and, in fact, gave the impression of being almost unavoidable.[48] Studies on cell lines during their evolution[94] and within heteroploid pop-

ulations[49,50] outlined the general selective mechanisms which seemed to follow a pattern of: diploidy-tetraploidy-heteroploidy-stem cell emergence-further selection of new stem cells.[48,72]

The quantitative approach to the study of cell lines in culture pioneered by Earle, Sanford, and their co-workers[26,103,104] was advanced and rapidly developed following the work of Puck and his associates,[88,89] and the ability of most heteroploid cell lines to grow under dispersed plating conditions has fostered much research involving clone derivations and the attempt to relate the characteristics of adapted or "mutant" cell lines to their karyologic content. Some examples of this were: virus resistance,[119] clumping factors in fresh serum,[101] and biochemical differences.[57]

Hsu[50] has taken a single well marked chromosome in the heterogeneous L cell and followed it alone, ignoring the 60-odd others, and has been able to correlate the frequency of this "D" marker with the conditions of culture. The proportion of D markers in the population under selection by "frequent" versus "less frequent" feeding schedules was subject to predictable shifts in spite of the crudeness of the cytological design which the L cell represents. In further studies, Hsu and Merchant[52] noted an iso-chromosome, apparently derived from the long arm of the D chromosome, which seemed to confer definite growth superiority to those sublines containing it.

The cloning of cell lines had already been practiced by Sanford, Likely, and Earle[103,104] and by Hauschka and Levan[41] with techniques providing a high assurance of the cell of origin but less adaptable to mass microbiological approaches. Five clonal lines derived by Puck were studied for their chromosomal constitutions by Chu and Giles[17] and in one instance there was a wide spread in chromosome numbers similar to the parental HeLa, but in the other four there were relatively uniform modes of the stem cell with slight spread to either side. However, the detailed cytology of the individual chromosomes was not extensive, and much more karyologic heterogeneity may be presumed to exist than is indicated by modal counts. This is, in fact, partly evident from their analyses of general centromeric types. Studies of the progeny of a single L cell by Hsu well illustrate this inherent variability of the typical cell line.[49]

In examining the chromosomal constitution of an AMK-2 subline of Lieberman's, which was selected for resistance to puromycin,[76] we have observed the deterioration of the original unimodal distribution in a period of about 14 days of culture in the absence of the selective force.

Although instability of cell division and variation of chromosome numbers and types through the resultant unequal segregation characterize the commonly available cell lines, it is not denied that chromosomal stability and consequently possible phenotypic stability could be achieved in some clones.[39] That every cell type in a parental or mixed population of a cell such as HeLa would necessarily be endowed with mitotic instability would be, in fact, surprising. Puck[88] has referred to these clonal derivative lines as "mutants" and although he states that this designation is not intended to imply the mechanism involved, its use is misleading considering the nearly limitless pool of

chromosomal variation directly available in a heteroploid line, and the fact that we are probably not far from dealing with "mutants" at the level of the gene locus in mammalian cells.

Similar usage of the term "mutant" has been applied to variant cell sublines or cloned lines by others.[69,70,114] Biochemical markers sought after in the above studies may permit the demonstration of DNA transformation in mammalian cells *in vitro* but unless relatively stable clones of a uniform karyologic background are used, such approaches are cytogenetically limited. Such stepwise selective changes noted in these heteroploid lines are most likely to represent variant chromosomal arrangements in the population, rather than gene mutations.

Roosa, Bradley, and Law[12,92] have derived various cloned lines from the P-388 mouse neoplastic cell line in which both "single step" and multiple small steps have been obtained during selection for resistance to various antimetabolites (amethopterin, 8-azaguanine, etc.). One clone appears to be relatively uniform in its chromosome content, offering some possibility for the further derivation of resistance markers from which gross chromosomal rearrangements may be excluded.[76]

Many of the problems brought out above have been encountered in the course of the well executed karyologic studies by Ruddle and Harris.[39,96] Starting with a relatively stable porcine line (PK-1), the diploid proportion being about 65 percent, a number of clones and subclones were established, apparently with little difficulty as to plating efficiency. Some fraction of the cells of the parental lines was definitely subtetraploid but has not overgrown the quasi-diploids although present in the cultures during subcultivation for five years. By X-raying, a number of marker translocations were obtained and followed extensively through clonings and subclonings. In some clones 95 percent diploidy was maintained and in others fluctuations toward hypodiploidy and increased heteroploidy were seen. As in the Chinese hamster diploid line of Yerganian,[126] a proportion of recognizably aberrant cells of hypodiploid and quasi-diploid type continues to be "thrown." Some clones derived from the original PK-1 parental lines became unstable heteroploid populations. Ruddle and Harris state that variant chromosomal types tend to increase gradually over periods of a year in culture. These authors have emphasized, as have Puck,[87] Tjio,[118] and Yerganian,[126] the practical necessity of periodic recloning. A point brought out in these studies[39,96] is that the cloning procedure itself subjects the cell to an exposed situation and may contribute to selection for heterogeneity. That is, we do not yet understand what a proper "diploid-favoring" optimal culture medium might be. We are ignorant of the degree of "force" we apply in demanding not only that our cells repeatedly divide, but that they surrender their normal differentiation plans and do not introduce mistakes in their mitoses.

In our laboratory we have, incidental to other purposes, obtained three lines which are similar to the PK-1 line in their karyologic pattern.[76] All three initially showed diploid mode but later were characterized by near-diploid

modes, a small percentage (5 to 10 percent) of cells with definitely abnormal chromosomes (dicentrics and rings) and increases in the percentage of tetraploids. Also, all three are epithelioid as is the PK-1. Two of these lines were derived from a rhesus monkey embryo (kidney and lung) and one from a calf embryo (kidney). In contrast to this, diploid human fibroblastic cell strains[44,87] do not show such alterations in the karyotype and show characteristically only about 2 to 3 percent tetraploidy. In our experience and that of others, such human fibroblast diploid cell strains cannot be cultivated indefinitely. The capacity for continuous cultivation would seem to be present in the PK-1, carried over 5 years, in Yerganian's line, and in our three epithelioid lines mentioned above. These are now in their second or more year, and show no decline in vigor.

In this comparison we should also consider possible species differences which may be quite important factors concerning *in vitro* mitotic stability. Rothfels and Parker[94] have raised this point in connection with a study of a number of mouse cell lines. The mouse's cells appear especially unstable *in vitro* as do the cells of the Chinese hamster.[126]

In the work of Harris and Ruddle and in many similar studies, although karyologic information comprised a major part of the work, the primary purpose was to determine if drug-resistant lines could be obtained and what their general chromosomal and growth characteristics might be. As Ruddle states, "We regard the chromosomes in this kind of study purely as a marker so that we can follow the fate of cells under various conditions. I think it would be presumptuous to regard them genetically in these studies. I might add we are designing systems which can be used to work out problems of true cytogenetic character."

In any case, in a study by Biesele, Biedler, and Hutchinson[9] a clear correlation *was* obtained. In studies of various drug-resistant lines of L-1210 leukemia they found the presence of a particular marker chromosome in each of four different sublines that were sensitive to amethopterin and in each of five sublines resistant to amethopterin this marker was absent. The finding of Nowell and Hungerford[79] that a specific chromosomal lesion is almost invariably associated with the condition of chronic granulocytic leukemia has helped to counter a too pessimistic view. We should sooner or later *expect* to find that the particular condition or phenotype studied does happen to have at least part of its underlying genetic basis within a chromosome that can be distinguished.

UNIT CHARACTERS IN CELL CULTURE

Our hopes for the development of combinations of techniques which will allow us to analyze cells for individual factors have been, of course, aroused by the striking successes of bacterial genetics involving transductive and transforming phenomena. The finding of suitable methods for hybridization and for segregation of the chromosomes of a genetically defined cell in culture would be quite important to fields such as cancer, immunology, and especially

differentiation. Schultz[107] has pointed out in his article, "Malignancy and the Genetics of the Somatic Cell," that the viral and mutational theories of cancerogenesis are not actually antithetical since the former reduces to a "special case of genetic interpretation of the neoplastic phenomenon." He proceeds to emphasize a more significant alternative, that "the malignant process is an aberration of the process of differentiation." This attitude directs our attention to the contrast between the sporadic nature of the mutational event and the orderly progression of differentiation. Until the nature of the series of differentiation states is better understood and its eventual control is obtained, our problems of clarity on *in vitro* phenotypic markers will perhaps be greater than the problem of cell mating or genetic transfer.

Except for the galactosemic cell strain studies,[61] the rather elementary level of our understanding to date is highlighted by the difficulties of obtaining diploid long-term cell cultures which do not rapidly lose their specific functional abilities.[37] For example, using the mixed agglutination reaction of Coombs and Bedford,[22] Högman[46] has found that human cells lose their capacity to react with antisera for the human A and B blood groups after 5 to 7 passages *in vitro*. This loss of a phenotypic trait is a gradual decrease in the proportion of reactive cells rather than a general reduction of avidity. The rapid loss of antigens *in vitro* has been demonstrated by Weiler.[120] Holtzer, Abbott, Lash, and Holtzer[47] have illustrated a similar loss of a cell capability in their studies of chick cartilage cells. After periods of a week under conditions *in vitro* favoring rapid growth, such cells are unable to synthesize chondroitin sulfate when returned to a standardized organ culture situation in which the primary cells *are* competent.

More optimistically we may expect certain embryological cell interactions and inductive phenomena as exemplified in the general approach of Wilde,[122] Moscona,[78] and others[106] to be exploited for use as end points or phenotypes.

In searching for workable methods, we should not overlook organ culture techniques. Individual or dispersed cell culture is an abstraction of biology offering great mental comfort to the reductionist personality but many functional capacities may be available to us which depend upon the "social" needs of cells. It was an understanding of this cell interplay in the idea of conditioned media[103] and the feeder layer[89] which yielded the first successful cloned cell lines.

In spite of the problem of loss of antigenic capacity, the antigen as a phenotypic marker is perhaps one of the most promising of all approaches to somatic cell genetics. Schultz,[108] Klein,[58] and Hauschka and Schultz[42] have discussed this problem extensively.

CELL "HYBRIDIZATION" OR FUSION

The exciting findings of Barski, Sorieul, and Cornefert[6] concerning *in vitro* cell fusions between cells of "high and low" cancer lines[105] may provide the much needed "missing link," a method for the introduction of genetic material from one mammalian cell into another. Such an approach has the advan-

tage of being physiologic or free of artifact. Sorieul and Ephrussi[109] have recently confirmed this phenomenon in different cell lines. In the work of Barski et al., two cloned lines having a common origin and known to yield "high" takes and "low" takes of cancers on injection into mice were used. These lines are heteroploid, having similar modes, N_1 at 55 and N_2 at both 58 and 62 chromosomes per cell. N_1's karyotype was entirely of telocentrics with one extra long telocentric. N_2 had about 12 metacentrics and the rest were telocentric. The new type of "hybrid" cell observed in mixed cultures had different biological properties but, more to the point, possessed a karyotype of 9 to 15 metacentrics of types not distinguishable from those of N_2, one extra long telocentric, and the remainder were telocentric. Altogether the modal chromosome number was approximately the sum of the two parental types. No such karyotypes were observed in repeated monitoring of the two parental populations, which should discount any autoploid mechanism. Each one of 279 metaphases from a cloned hybrid tumor cultured *in vitro* was of the "hybrid" karyotype. An interesting point in their work was the rather high frequency of the occurrence of cell fusion and some of their evidence indicated a selective advantage for the "hybrid" cell. This should give repeated opportunities to study the event itself with an eye to enhancing the frequency of fusion or of inducing fusion in other cells. In the large film collection of cinematographic phase contrast records of HeLa and of many other cell types compiled by Pomerat (which I have had the opportunity to examine), no certain example of fusion was ever noted. However, in experiments[76] involving primary cultures of rabbit spleen and lymph nodes, as many as 12 different direct cell fusions were filmed in several cultures within a relatively short period. These fusions led to the formation of giant cells with dozens of nuclei, as is typical of cell response to certain viruses.[45,82,91] However, none of such fused giant cells was ever observed to divide. In binucleate HeLa cells both nuclei proceed from prophase into metaphase simultaneously.[77]

Once cell mating has been achieved, can we expect synapsis of homologues and somatic crossing over? This subject has been ably considered by Klein[58] in relation to certain antigenic results obtained in studies of the induction of tumors in F_1 mouse hybrids (apparent changes in antigenic loci) which suggested somatic crossing over as an explanation. Somatic crossing over has been known to exist in Drosophila since Stern's classic "twin-spot" proof.[111] In Wilson's work,[123] *The Cell in Development and Heredity,* several authors are cited who produced evidence from metaphase observations that homologous chromosomes occasionally tended to reveal striking affinities or associations. Most workers examining metaphases from human peripheral blood or "fibroblast" preparations have noted examples of apparent homologue association or pairing in spite of the flattening of the metaphase group by squashing or by air drying. Statistical evidence has been obtained in support of somatic metaphase association by Ruddle.[97] Boss[10] has compiled a short review of the reports of somatic pairing and has drawn his own observations supporting its occurrence primarily from newt tissue cultivated *in vitro*. Photo-

graphs of metaphases interpretable as either strong metaphase pairing or the products of a reduction division in tissue cultures of human cells have been published.[102]

Pontecorvo,[84] in his article, "Microbial Genetics and Human Genetics," has belittled the feeling that proof of somatic cell crossing over is necessary before we can hope to make use of cell fusion and ordinary mitotic segregations in tissue-cultured material. His work with the "parasexual cycle" of Aspergillus is presented by him as a model illustrating the considerable amount of genetic information possibly obtainable from cultures of mammalian cells. From mitotic nondisjunction alone, information on linkage can be extracted. For example, Pontecorvo points out that beginning with a diploid heterozygous for various alleles we might obtain a trisomic which upon further occurrence of nondisjunction, eliminating the odd member of the chromosome trio, would yield a diploid homozygous for all the alleles of that chromosome while remaining unaltered at all other chromosome loci.

Although possible, the difficulties of this segregational recovery of previously mixed alleles would seem formidable. Pontecorvo has expressed the view that repeated loss of chromosomes in steps could be fostered by the possibly more desirable "balance" of the haploid as against the sub-diploid condition and that the large amount of nondisjunction occurring in mammalian cultures would be sufficient for this. Except for fortuitous reductions of chromosome number in heteroploids, the only immediate examples of "stripping off" of chromosomes are those seen after high doses of radiation.[90] Chance selection appears more likely to proceed to increases than to decreases in the chromosome complement unless one begins with material which is hyperdiploid.

A possibility worth attention may be to attempt the combination of the techniques such as Briggs and King[14] have used involving nuclear transplantation by micromanipulation and the culture of cells from haploid organisms as reported by Freed and Cole.[33] Haploid cell cultures from haploid frogs are reported by these workers to revert rapidly to diploidy. Although perhaps too little effort has yet been invested in such feats, it may be that cells of animals of lower orders would prove more easily managed in culture as haploids for the eventual direct production of heterokaryons. Parthenogenetic turkeys have been developed, but these unfortunately also revert to diploidy.[86] Such organisms have apparently an auto-diploid constitution which results from a tendency to fusion during the egg's early development.

As ubiquitous as cell, tissue, and organ culture methodology seems to be in research, there are no doubt many interesting untried possibilities. A better understanding of the physiology of mitosis and meiosis,[73] it is hoped, may open opportunities for the eventual manipulation of somatic segregation and haploidization in culture. However, at present, it seems that the mammalian diploid cell is best adapted to the body, the heteroploid cell to *in vitro* cultivation, and the haploid cell to a gonadal tissue environment.

REFERENCES

1. Allen, G. (Ed.): Cytology and Cell Culture Genetics of Man. Symposium. Am. J. Human Genet. *12*:97–138, 1960.
2. Apgar, V.: Human Congenital Anomalies. A.M.A. J. Dis. Children *101*:249–254, 1961.
3. Aronson, M., and Elberg, S. S.: Fusion of Peritoneal Histiocytes with Formation of Giant Cells. Nature *193*:399, 1962.
4. Barnes, D. W. H.; Ford, C. E.; Gray, S. M.; and Loutit, J. F.: Spontaneous and Induced Changes in Cell Populations in Heavily Irradiated Mice. Progr. Nuclear Energy, Biol. Sc. *2*:1–10, 1959.
5. Barr, M. L., and Carr, D. H.: Correlations Between Sex Chromatin and Sex Chromosomes. Acta Cytol. *6*:34–45, 1962.
6. Barski, G.; Sorieul, S.; and Cornefert, Fr.: "Hybrid" Type Cells in Combined Cultures of Two Different Mammalian Cell Strains. J. Nat. Cancer Inst. *26*:1269–1291, 1961.
7. Bayreuther, K.: The Chromosomal Constitution of Methyl-Cholanthrene Induced Sarcomas and Their Variants Induced and Tested in Isogenic Resistant Strains. Acta Unio internat. cancr., Louvain *16*:55–58, 1960.
8. Berman, L., and Stulberg, C. S.: The Detroit Strains of Human Epithelial-like Cells from Nonleukemic Peripheral Blood. Blood *13*:1149–1167, 1958.
9. Biesele, J. J.; Biedler, J. L.; and Hutchinson, D. J.: The Chromosomal Status of Drug Resistant Sublines of Mouse Leukemia L 1210. In *Genetics and Cancer*. Austin: University of Texas Press, 1959, pp. 295–307.
10. Boss, J. M. N.: The Pairing of Somatic Chromosomes: A Survey. Texas Rep. Biol. M. *13*:213–221, 1955.
11. Boveri, T., cited by Wilson, E. B.: *The Cell in Development and Heredity,* 3rd ed. New York: The Macmillan Company, 1925, pp. 890–892.
12. Bradley, T. R.; Roosa, R. A.; and Law, L. W.: DNA Transformation Studies with Mammalian Cells Grown in Culture. J. Cellul. Physiol. (in press).
13. Brand, K. G., and Syverton, J. T.: Results of Species -Specific Hemagglutination Tests on "Transformed," Nontransformed, and Primary Cell Cultures. J. Nat. Cancer Inst. *28*:147–157, 1962.
14. Briggs, R., and King, T. J.: Changes in the Nuclei of Differentiating Endoderm Cells as Revealed by Nuclear Transplantation. J. Morphol. *100*:269–311, 1957.
15. Carter, T. C.; Lyon, M. F.; and Phillips, R. J. S.: Gene-Tagged Chromosome Translocations in Eleven Stocks of Mice. J. Genet. *53*:154–166, 1955.
16. Chang, R. S.: Isolation of Nutritional Variants from Conjunctival and HeLa Cells. Proc. Soc. Exp. Biol. *96*:818–820, 1957.
17. Chu, E. H. Y., and Giles, N. H.: Comparative Chromosomal Studies on Mammalian Cells in Culture; I. The HeLa Strain and Its Mutant Clonal Derivatives. J. Nat. Cancer Inst. *20*:383–396, 1957.
18. Chu, E. H. Y., and Giles, N. H.: Chromosome Complements and Nucleoli in Normal Human Somatic Cells in Culture (Abstract). X Internat. Cong. Genet., Montreal *11*:50, 1958.
19. Chu, E. H. Y., and Giles, N. H.: Human Chromosome Complements in Normal Somatic Cells in Culture. Am. J. Human Genet. *11*:63–79, 1959.
20. Chu, E. H. Y.; Sanford, K. K.; and Earle, W. R.: Comparative Chromosomal Studies on Mammalian Cells in Culture; II. Mouse Sarcoma-Producing Cell Strains and Their Derivatives. J. Nat. Cancer Inst. *21*:729–751, 1958.
21. Clausen, J. J., and Syverton, J. T.: Comparative Chromosomal Study of 31 Cultured Mammalian Cell Lines. J. Nat. Cancer Inst. *28*:117–145, 1962.
22. Coombs, R. R. A., and Bedford, D.: The A and B Antigens on Human Platelets Demonstrated by Means of Mixed Erythrocyte-Platelet Agglutination. Vox sanguinis, Basel *5*:111–115, 1955.
23. Coriell, L. L.; Tall, M. G.; and Gaskill, H.: Common Antigens in Tissue Culture Cell Lines. Science *128*:198–199, 1958.
24. Defendi, V.; Billingham, R. E.; Silvers, W. K.; and Moorhead, P. S.: Immunological and Karyological Criteria for Identification of Cell Lines. J. Nat. Cancer Inst. *25*:359–385, 1960.
25. Dickie, M. M.: The Expanding Knowledge of the Genome of the Mouse. J. Nat. Cancer Inst. *15*:679–684, 1954.

26. Earle, W.; Schilling, E. L.; Stark, T. H.; Straus, N.; Brown, M. F.; and Shelton, E.: Production of Malignancy *in vitro;* IV. The Mouse Fibroblast Cultures and Changes Seen in Living Cells. J. Nat. Cancer Inst. *4*:165–212, 1943.
27. Ford, C. E.: Human Cytogenetics: Its Present Place and Future Possibilities. Am. J. Human Genet. *12*:104–117, 1960.
28. Ford, C. E., and Hamerton, J. L.: A Colchicine Hypotonic Citrate Squash Sequence for Mammalian Chromosomes. Stain Techn. *31*:247–251, 1956.
29. Ford, C. E.; Hamerton, J. L.; and Mole, R. H.: Chromosomal Changes in Primary and Transplanted Reticular Neoplasms of the Mouse. J. Cellul. Physiol. *52*:235–269, 1958.
30. Ford, C. E.; Ilbery, P. L. T.; and Loutit, J. F.: Further Cytological Observations on Radiation Chimeras. J. Cellul. Physiol. *50*:109–121, 1957.
31. Ford, D. K.; Wakonig, R.; and Yerganian, G.: Further Observations on the Chromosomes of Chinese Hamster Cells in Tissue Culture. J. Nat. Cancer Inst. *22*:765–799, 1959.
32. Foulds, L.: The Natural History of Cancer. J. Chronic Dis. *8*:2–37, 1958.
33. Freed, J. J., and Cole, S. J.: Chromosome Studies on Haploid and Diploid Cell Cultures from *Rana pipiens* (Abstract). First Annual Meeting, American Society for Cell Biology, Chicago, 1961, p. 62.
34. Geitler, L., cited by Swanson, C. P.: In *Cytology and Cytogenetics.* Englewood Cliffs, N. J.: Prentice-Hall, Inc., 1957, p. 297.
35. Gey, G. O.; Coffman, W. D.; and Kubicek, M. T.: Tissue Culture Studies of the Proliferative Capacity of Cervical Carcinoma and Normal Epithelium (Abstract). Cancer Res. *12*:264–265. 1952.
36. Griffen, A. B.: Mammalian Cytogenetics and the Cancer Problem. Ann. N. York Acad. Sc. *71*:1156–1162, 1958.
37. Grobstein, C.: Differentiation of Vertebrate Cells. In Brachet, J., and Mirsky, A. E. (Eds.): *The Cell,* vol. 1. New York: Academic Press, Inc., 1959, pp. 437–496.
38. Harnden, D. G.: Nuclear Sex in Triploid XXY Human Cells. Lancet *2*:488, 1961.
39. Harris, M., and Ruddle, F. H.: Clone Strains of Pig Kidney Cells with Drug Resistance and Chromosomal Markers. J. Nat. Cancer Inst. *26*:1405–1411, 1961.
40. Hauschka, T. S.: Tissue Genetics of Neoplastic Cell Populations. Canad. Cancer Conf. *2*:305–345, 1957.
41. Hauschka, T. S., and Levan, A.: Cytologic and Functional Characterization of Single Cell Clones Isolated from the Krebs-2 and Ehrlich Ascites Tumors. J. Nat. Cancer Inst. *21*:77–135, 1958.
42. Hauschka, T. S., and Schultz, J.: Cytologic Aspects of Immunogenetic Specificity. Transpl. Bull. *1*:203–206, 1954.
43. Hay, J. C., and Moore, K. L.: The Sex Chromatin in Various Mammals. Acta anat., Basel *45*:289–309, 1961.
44. Hayflick, L., and Moorhead, P. S.: The Serial Cultivation of Human Diploid Cell Strains. Exp. Cell Res. *25*:585–621, 1961.
45. Henle, G.; Deinhardt, F.; and Girardi, A.: Cytologic Effects of Mumps Virus in Tissue Cultures of Epithelial Cells. Proc. Soc. Exp. Biol. *87*:386–393, 1954.
46. Högman, C. F.: Blood Group Antigens on Human Cells in Tissue Culture. Exp. Cell Res. *21*:137–143, 1960.
47. Holtzer, H.; Abbott, J.; Lash, J.; and Holtzer, S.: The Loss of Phenotypic Traits by Differentiated Cells *in vitro;* I. Dedifferentiation of Cartilage Cells. Proc. Nat. Acad. Sc. *46*:1533–1542, 1960.
48. Hsu, T. C.: Chromosomal Evolution in Cell Populations. Internat. Rev. Cytol. *12*:69–161, 1961.
49. Hsu, T. C.: Mammalian Chromosomes *in vitro;* XI. Variability among Progenies of a Single Cell. University of Texas Publication No. 5914, 1959, pp. 129–134.
50. Hsu, T. C.: Mammalian Chromosomes *in vitro;* XIII. Cyclic and Directional Changes of Population Structure. J. Nat. Cancer Inst. *25*:1339–1353, 1960.
51. Hsu, T. C., and Klatt, O.: Mammalian Chromosomes *in vitro;* IX. On Genetic Polymorphism in Cell Populations. J. Nat. Cancer Inst. *21*:437–473, 1958.
52. Hsu, T. C., and Merchant, D. J.: Mammalian Chromosomes *in vitro;* XIV. Genotypic Replacement in Cell Populations. J. Nat. Cancer Inst. *26*:1075–1081, 1961.

53. Hsu, T. C., and Moorhead, P. S.: Mammalian Chromosomes *in vitro;* VII. Heteroploidy in Human Cell Strains. J. Nat. Cancer Inst. *18*:463–471, 1957.
54. Hsu, T. C., and Pomerat, C. M.: Mammalian Chromosomes *in vitro;* II. A Method for Spreading the Chromosomes of Cells in Tissue Culture. J. Hered. *44*:23–29, 1953.
55. Hsu, T. C., and Somers, C. E.: Effect of 5-Bromodeoxyuridine on Mammalian Chromosomes. Proc. Nat. Acad. Sc. *47*:396–402, 1961.
56. Hungerford, D. A.: Chromosome Numbers of Ten-Day Fetal Mouse Cells. J. Morphol. *97*:497–510, 1955.
57. Kit, S.; Fiscus, J.; Ragland, R. S.; Graham, O. L.; and Gross, A. L.: Biochemical Studies on the Chinese Hamster (22 Chromosomes) and the Syrian Hamster (44 Chromosomes). Exp. Cell Res. *16*:411–417, 1959.
58. Klein, G.: Somatic Cell Genetics. In *Methodology in Mammalian Genetics.* San Francisco: Holden-Day, Inc., 1962.
59. Klinger, H. P., and Schwartzacher, H. G.: The Sex Chromatin and Heterochromatic Bodies in Human Diploid and Polyploid Nuclei. J. Biophys. Cytol. *8*:345–364, 1960.
60. Koller, P. C.: Cytological Variability in Human Carcinomatosis. Ann N. York Acad. Sc. *63*:793–816, 1956.
61. Krooth, R. S., and Weinberg, A. N.: Studies on Cell Lines Developed from the Tissues of Patients with Galactosemia. J. Exp. M. *113*:1155–1172, 1961.
62. Lederberg, J.: Prospects for a Genetics of Somatic and Tumor Cells. Ann. N. York Acad. Sc. *63*:662–665, 1956.
63. Leighty, R. W., and Plaisted, R. L.: The Evaluation of the Sources of Variation in the Preparation of a Karyotype of a Clone. Cytologia *25*:1–7, 1960.
64. Lejeune, J., and Turpin, R.: Chromosomal Aberrations in Man. Am. J. Human Genet. *13*:175–184, 1961.
65. Lejeune, J., and Turpin, R.: Detection Chromosomique d'une Mosaique Artificielle Humaine. C. rend. Acad. Sc. *252*:3148–3150, 1961.
66. Lettre, H.: Ergebnisse und Probleme der Mitosegiftforschung. Naturwiss. *33*:117, 1946.
67. Levan, A.: Chromosome Studies on Some Human Tumors and Tissues of Normal Origin, Grown *in vivo* and *in vitro* at the Sloan-Kettering Institute. Cancer *9*:648, 1956.
68. Levan, A., and Hauschka, T. S.: Chromosome Numbers of Three Mouse Ascites Tumours. Hereditas *38*:251–255, 1952.
69. Lieberman, I., and Ove, P.: Isolation and Study of Mutants from Mammalian Cells in Culture. Proc. Nat. Acad. Sc. *45*:867–872, 1959.
70. Lieberman, I., and Ove, P.: Estimation of Mutation Rates with Mammalian Cells in Culture. Proc. Nat. Acad. Sc. *45*:872–877, 1959.
71. Longwell, A. C., and Svihla, G.: Specific Chromosomal Control of the Nucleolus and of the Cytoplasm in Wheat. Exp. Cell Res. *20*:294–312, 1960.
72. Makino, S.: The Concept of Stemline-Cells as Progenitors of a Neoplastic Population. Cytologia, Suppl. Vol. (Proc. Internat. Genet. Sympos., 1956), 1957, pp. 177–181.
73. Mazia, D.: Mitosis and the Physiology of Cell Division. In Brachet, J. (Ed.): *The Cell,* vol. 3. New York: Academic Press, Inc., 1961.
74. McCulloch, E. A.; Parker, R. C.; and Wightman, K. J. R.: Continuous Cultivation of Cells Derived from Hemic Cells of Man and Pure Strain Mice (Abstract). Proc. Am. Assn. Cancer Res. *2*:132, 1956.
75. Micklem, H. S., and Ford, C. E.: Proliferation of Injected Lymph Node and Thymus Cells in Lethally Irradiated Mice. Transpl. Bull. *26*:436–441, 1960.
76. Moorhead, P. S.: Unpublished.
77. Moorhead, P. S., and Hsu, T. C.: Cytologic Studies of HeLa, a Strain of Human Cervical Carcinoma; III. Durations and Characteristics of the Mitotic Phases. J. Nat. Cancer Inst. *16*:1047–1066, 1956.
78. Moscona, A.: The Development *in vitro* of Chimeric Aggregates of Dissociated Embryonic Chick and Mouse Cells. Proc. Nat. Acad. Sc. *43*:184–194, 1957.
79. Nowell, P. C., and Hungerford, D. A.: Chromosome Studies in Human Leukemia. II. Chronic Granulocytic Leukemia. J. Nat. Cancer Inst. *27*:1013–1035, 1961.
80. Ohno, S., and Hauschka, T. S.: Allocycly of the X-Chromosome in Tumors and Normal Tissues. Cancer Res. *20*:541–545, 1960.
81. Ohno, S.; Kovacs, E. T.; and Kinosita, R.: A Robertsonian Type of Chromosomal Change in L4946 Mouse Ascites Lymphoma. J. Nat. Cancer Inst. *24*:1187–1197, 1960.

82. Okada, Y.; Suzuki, T.; and Hosaka, Y.: Interaction Between Influenza Virus and Ehrlich's Tumor Cells; III. Fusion Phenomenon of Ehrlich's Tumor Cells by the Action of HVJ Z Strain. Med. J. Osaka Univ. *7*:709–717, 1957.
83. Patau, K.: The Identification of Individual Chromosomes, Especially in Man. Am. J. Human Genet. *12*:250–276, 1960.
84. Pontecorvo, G.: Microbiol Genetics and Human Genetics. Brit. M. Bull. *18*:81–84, 1962.
85. Ponten, J.: Homologous Transfer of Rous Sarcoma by Cells. J. Nat. Cancer Inst. (in press).
86. Poole, H. K.: The Mitotic Chromosomes of Parthenogenetic and Normal Turkeys. J. Hered. *50*:151–154, 1959.
87. Puck, T. T.; Cieciura, S. J.; and Robinson, A.: Genetics of Somatic Mammalian Cells; III. Long-Term Cultivation of Euploid Cells from Human and Animal Subjects. J. Exp. M. *108*:945–956, 1958.
88. Puck, T. T., and Fisher, H. W.: Genetics of Somatic Mammalian Cells; I. Demonstration of the Existence of Mutants with Different Growth Requirements in a Human Cancer Cell Strain (HeLa). J. Exp. M. *104*:427–434, 1956.
89. Puck, T. T., and Marcus, P. I.: A Rapid Method for Viable Cell Titration and Clone Production with HeLa Cells in Tissue Culture: The Use of X-irradiated Cells to Supply Conditioning Factors. Proc. Nat. Acad. Sc. *41*:432–437, 1955.
90. Rhynas, P. O. W., and Newcombe, H. B.: A Heritable Change in Radiation Resistances of Strain L Mouse Cells. Exp. Cell Res. *21*:326–331, 1960.
91. Roizman, B.: Virus Infection of Cells in Mitosis; I. Observations on the Recruitment of Cells in Karyokinesis into Giant Cells Induced by Herpes Simplex Virus, and Bearing on the Site of Virus Antigen Formation. Virology *13*:387–401, 1961.
92. Roosa, R. A.; Bradley, T. R.; Law, L. W.; and Herzenberg, L. A.: Characterization of Resistance to Amethopterin, 8-Azaguanine and Fluorinated Pyrimidines in the Mouse Lymphocytic Neoplasm, P-388, in Cell Culture. J. Cellul. Physiol. (in press).
93. Rothfels, K. H.; Axelrad, A. A.; Siminovitch, L.; McCulloch, E. A.; and Parker, R. C.: The Origin of Altered Cell Lines from Mouse, Monkey and Man, as Indicated by Chromosome and Transplantation Studies. Canad. Cancer Conf. *3*:189–214, 1959.
94. Rothfels, K. H., and Parker, R. C.: The Karyotypes of Cell Lines Recently Established from Normal Mouse Tissues. J. Exp. Zool. *142*:507–520, 1959.
95. Rothfels, K. H., and Siminovitch, L.: An Air-Drying Technique for Flattening Chromosomes in Mammalian Cells Grown *in vitro*. Stain Techn. *33*:73, 1958.
96. Ruddle, F. H.: Chromosome Variation in Cell Populations Derived from Pig Kidney. Cancer Res. *21*:885–894, 1961.
97. Ruddle, F. H.: Personal communication.
98. Ruddle, F. H.; Berman, L.; and Stulberg, C. S.: Chromosome Analysis of Five Long-Term Cell Culture Populations Derived from Non-Leukemic Human Peripheral Blood (Detroit Strains). Cancer Res. *18*:1048–1059, 1958.
99. Russell, L. B., and Chu, E. H. Y.: An XXY Male in the Mouse. Proc. Nat. Acad. Sc. *47*:571–575, 1961.
100. Russell, W. L.; Russell, L. B.; and Gower, J. S.: Exceptional Inheritance of a Sex-Linked Gene in the Mouse Explained on the Basis That the X/O Sex Chromosome Constitution Is Female. Proc. Nat. Acad. Sc. *45*:554–560, 1959.
101. Saksela, E.; Saxen, E.; and Penttinen, K.: Chromosome Heterogeneity of Cell Lines *in vitro;* I. Differences in the Effect of Individual Human Sera on the Chromosome Number of HeLa Cells. Acta path. microb. scand. *51*:127–131, 1961.
102. Sandberg, A. A.; Koepf, G. E.; Grosswhite, L. H.; and Hauschka, T. S.: The Chromosome Constitution of Human Marrow in Various Developmental and Blood Disorders. Am. J. Human Genet. *12*:231–249, 1960.
103. Sanford, K. K.; Covalesky, A. B.: Dupree, L. T.; and Earle, W. R.: Cloning of Mammalian Cells by a Simplified Capillary Technique. Exp. Cell Res. *23*:361–372, 1961.
104. Sanford, K. K.; Likely, G. D.; and Earle, W. R.: The Development of Variations in Transplantability and Morphology Within a Clone of Mouse Fibroblasts Transformed to Sarcoma-Producing Cells *in vitro*. J. Nat. Cancer Inst. *15*:215–237, 1954.
105. Sanford, K. K.; Merwin, R. M.; Hobbs, G. L.; Young, K. M.; and Earle, W. R.: Clonal Analysis of Variant Cell Lines Transformed to Malignant Cells in Tissue Culture. J. Nat. Cancer Inst. *23*:1035–1059, 1959.

106. Saxen, L.; Toivonen, S.; and Vainio, T.: The Embryonic Inductive Action of Cells Cultivated *in vitro*. Acta path. microb. scand. *51*(Suppl. 144):163–164, 1961.
107. Schultz, J.: Malignancy and the Genetics of the Somatic Cell. Ann. N. York Acad. Sc. *71*:994–1008, 1958.
108. Schultz, J.: Antigens and Antibodies as Cell Phenotypes. Science *129*:937–943, 1959.
109. Sorieul, S., and Ephrussi, B.: Karyological Demonstration of Hybridization of Mammalian Cells *in vitro*. Nature *190*:653–654, 1961.
110. Stern, C.: Zytologisch-genetische Untersuchungen als Beweise fur die Morganische theorie des factorenaustauschs. Biol. Zbl. *51*:547–587, 1931.
111. Stern, C.: Somatic Crossing over and Segregation in *Drosophila melanogaster*. Genetics *21*:625–730, 1936.
112. Stich, H. F.: Chromosomes of Tumor Cells; I. Murine Leukemias Induced by One or Two Injections of 7, 12-Dimethylbenz (a) Anthracene. J. Nat. Cancer Inst. *25*:649–661, 1960.
113. Stich, H. F., and Hsu, T. C.: Cytological Identification of Male and Female Somatic Cells in the Mouse. Exp. Cell Res. *20*:248–249, 1960.
114. Szybalski, W., and Smith, M. J.: Genetics of Human Cell Lines; I. 8-Azaguanine Resistance, a Selective "Single Step" Marker. Proc. Soc. Exp. Biol. *101*:662–666, 1959.
115. Tjio, J. H., and Levan, A.: Chromosome Analysis of Three Hyperdiploid Ascites Tumours of the Mouse. Lunds Universitets Arsskrift, N. F. Avd. 2, Bd. 50, Lunds, 1954, 38 pp.
116. Tjio, J. H., and Levan, A.: The Chromosome Number of Man. Hereditas *42*:1–6, 1956.
117. Tjio, J. H., and Puck, T. T.: The Somatic Chromosomes of Man. Proc. Nat. Acad. Sc. *44*:1229–1237, 1958.
118. Tjio, J. H., and Puck, T. T.: Genetics of Somatic Mammalian Cells; II. Chromosomal Constitution of Cells in Tissue Culture. J. Exp. M. *108*:259–268, 1958.
119. Vogt, M.: A Study of the Relationship Between Karyotype and Phenotype in Cloned Lines of Strain HeLa. Genetics *44*:1257–1270, 1959.
120. Weiler, E.: Loss of Specific Cell Antigen in Relation to Carcinogenesis. In Wolstenholme, G. W. E., and O'Connor, M. (Eds.): *Ciba Foundation Symposium on Carcinogenesis, Mechanism of Action*. Boston: Little, Brown and Company, 1958, pp. 165–178.
121. Westwood, J. C. N., and Titmuss, D. H. J.: Transformation in Tissue Culture Cell Lines; The Possible Genetic Mechanism. Brit. J. Exp. Path. *38*:587–600, 1957.
122. Wilde, C. E.: Differentiation in Response to the Biochemical Environment. In Rudnick, D. (Ed.): *Cell, Organism and Milieu*. New York: The Ronald Press Company, 1959, pp. 3–43.
123. Wilson, E. B.: *The Cell in Development and Heredity*, 3rd ed. New York: The Macmillan Company, 1925, pp. 836–838.
124. Woodruff, M. F. A.; Fox, M.; Buckton, K. A.; and Jacobs, P. A.: The Recognition of Human Blood Chimaeras. Lancet *1*:192–194, 1962.
125. Yerganian, G.: Chromosomes of the Chinese Hamster, *Cricetulus griseus;* I. The Normal Complement and Identification of Sex Chromosomes. Cytologia *24*:66–75, 1959.
126. Yerganian, G., and Leonard, M. J.: Maintenance of Normal *in situ* Chromosomal Features in Long-Term Tissue Cultures. Science *133*:1600–1601, 1961.

Biochemical and Physiological Markers

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GENETICS is concerned with the transmission of characteristics from parents to the offspring, and with the origin of variation within a discrete population of organisms. Currently, geneticists are interested in the storage and replication of genetic information,[1] the coding and translation of genetic information into physiological activity,[2–4] the interactions among germ plasm, cytoplasm, and environment,[5] and the mechanisms for genetic variation.[6] The present discussion is limited to the consideration of biochemical characterization of cells in continuous culture and the methodology for detecting gene recombination among physiological markers.

Strain identification and genetic analyses cannot be carried out unless certain cell lines differ overtly one from another. For dissimilar activities to be reliable for characterization of sublines or cells of diverse origin, the observations and comparisons must be made under identical conditions. Power of one mammalian cell culture to grow in horse serum-sucrose medium and inability of another culture to grow in human serum-sucrose medium does not mean that the two strains differ with respect to invertase activity. Indeed, horse serum possesses invertase activity whereas human serum lacks this enzyme.[7] Diagnosis of the phenotype of given strains may yield disparate results even when the same medium is used. The population density of newly inoculated cells is a critical factor for initiating growth.[8] Similarly, age of the culture is a determinative factor; human esophageal epithelial cells in the exponential phase of growth are killed by 10 μg. cycloheximide/ml., whereas metabolically inactive cells survive in the presence of 15 μg. antibiotic/ml.[9]

Conduct of comparisons between cell lines under the same conditions does not ensure that discrepant behavior reflects a fundamental genetic difference between two cultures. A population of *Escherichia coli,* for example, grown in the presence of succinate and methyl-thiogalactoside develops the enzyme β-galactosidase. Bacteria grown in succinate medium without methyl-thiogalactoside remain deficient for the enzyme. The induced culture, transferred to medium containing methyl-thiogalactoside and glucose, continues to make enzyme but uninduced bacteria in this medium cannot adapt.[10] The past experience of the two cultures has resulted in disparate expressions which are maintained indefinitely in medium containing both inducer and inhibitor. It is clear that the expressions of a cell are the result of interactions involving the genetic complement, the immediate environment, and the physiological states determined by previous conditions.

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For a characteristic to have maximal utility for genetic studies, it should be easily and accurately recognizable, be stable, be subject to selection, and should not affect growth rate or viability. The genetic control of a mutant phenotype should be relatively straightforward and its physiological interactions partially elucidated. To date, instability of particular phenotypes probably has been the most serious limitation for the genetic analysis of mammalian cell-culture systems. Much of the variability in expression can be attributed to inconsistency of the chromosomal content of diverse nuclei in a population. The number of chromosomes in nuclei of a given line of mammalian cells in continuous culture varies greatly.[11] Most established human cell strains contain 65 to 80 chromosomes,[12] which are greatly in excess of the diploid number of 46.[13] Most established cell cultures are aneuploid (heteroploid), that is, the nuclei do not contain only complete sets of chromosomes. Nuclei with one or more chromosomes added to one of the complete sets are known as polysomic and those missing one or more chromosomes of a set are hyposomic. A given aneuploid nucleus may be polysomic with respect to certain chromosomes and hyposomic with respect to other chromosomes. In contrast, euploid nuclei have complete sets of genetic information with no extra or lost chromosomes. A haploid nucleus contains one set of chromosomes which as a whole constitutes one complete set of genetic information (one genome), a diploid nucleus contains two sets of chromosomes, and a triploid nucleus has three sets of chromosomes. The term, polyploidy, refers to the euploid state but is often erroneously used to describe cell cultures. Diagnosis of the state of ploidy of a nucleus cannot be made solely on the basis of chromosome counts. Chromosomal morphology, that is, the length of the arms, their width, and the placement of the centromere, can be used for identifying groups of chromosomes.[14] Because recognition of each chromosome in the human cell is not yet possible, euploidy cannot be definitively demonstrated. Frequently, the aneuploid state of a nucleus can be readily established.

The phenotype of a cell is dependent not only upon the kinds of genes present but upon the gene dose. The cubitus vein of the wing of the fruit fly *Drosophila melanogaster* is markedly reduced in the diploid organism which is homozygous, for the mutant allele *cubitus interruptus (ci)*. A fly which has an allele for *cubitus interruptus* unopposed by another allele (hemizygous) possesses little or no cubitus wing vein. If a fly possesses three *ci* genes (trisomic) then the wing vein is nearly normal.[15] Insertion of additional mutant alleles re-establishes the normal phenotype! Compounding of mutant genes does not necessarily lead to an approximation of the wild-type expression. In Drosophila, the eye of a normal fly has about 750 facets whereas a (male) fly which is hemizygous (1 dose) for *infra-bar* contains about 475 facets per eye and a fly which is a homozygous diploid (2 doses) for *infra-bar* has only 300 facets. Drosophila with the duplication *infra-bar* on one chromosome, and a double duplication on the homologous chromosome (3 doses) produces eyes with only 150 facets. Finally, eyes of flies with double duplications on both X-chromosomes have only 40 facets.[16] Increasing the gene dosage of a

mutant allele may well lead to a stronger expression of the mutant phenotype.

To determine if two strains which appear different are genetically dissimilar, it is necessary to carry out a heritability test.[17] For example, a cell line which is able to grow in either a high concentration or a low concentration of glucose can be compared with a cell line that grows in the glucose-rich medium only. Both strains are subcultured serially in high-sugar medium; the glucose-frugal strain is also grown in low-sugar medium. After several transfers, each culture is inoculated into both the glucose-rich and glucose-poor media. If the glucose-frugal strain has been derived by nonhereditary *training* or adaptation, cultivation in high-glucose medium will bring about *deadaptation* or loss of power to initiate good growth in low-glucose medium. Power of hereditary variants to initiate growth in particular alternative substrates is not attenuated by growth in other nutritionally sufficient media.[18]

GENETIC MARKERS

Hereditary variants of mammalian cells may arise by gene mutation or by nuclear reorganization within cells of established cultures or may be derived from whole animals having distinct metabolic differences. Because most gene mutations are recessive, all the chromosomes bearing a given locus must contain the same alteration if a cell is to express the new phenotype. Assuming a mutation rate of once per 10^5 gene replications, the probability of establishing a mutant, homozygous diploid cell in one step from homozygous normal tissue by gene mutation alone is 10^{-10} and the chance of establishing a homozygous trisomic cell is 10^{-15}. Under these conditions, it would not usually be possible to obtain large enough populations of mammalian cells in continuous culture to use direct selection as a source of variants homozygous for new gene mutations. However, initial heterozygosity and incomplete dominance of mutant genes might alter these low probabilities and permit derivation of altered cell lines from clonal populations. Most of the hereditary variants derived from an established cell line have differed from the parental strain with respect to carbohydrate[19] or amino acid requirements,[20] drug susceptibility,[21] and enzymic activity.[22] The major difficulty with markers based upon power of cells to grow in sugar media with a carbohydrate other than glucose has been preparation of glucose-free media. Some workers have relied upon dialyzed serum and commercially available carbohydrates[23]; such media frequently contain several hundred micrograms of glucose per milliliter. Glucose in these media is derived not only from that contaminating particular carbohydrates, but also from dialyzed serum itself.[24] Although hereditary variants seem to have been obtained by direct selection in xylose medium, ribose medium, and galactose medium,[18,19] the alteration is not for specific, augmented power to utilize these sugars, but for increased ability to grow with low concentrations of glucose.[25] Xylose-selected cells and galactose-selected cells grow equally well in media containing either of these sugars! Attempts to demonstrate increased utilization of the particular carbohydrates have been unconvincing. Deficiencies in experiments using uptake of C^{14}-sugar as an index of utilization have been:

(a) use of several hundred micrograms of radioactive sugar whose purity has not been critically determined, (b) use of excessive amounts of labeled sugar, and (c) failure to give data on absolute uptake and recovery of the isotope. Moreover, the ability of 2-deoxyglucose at a concentration of 0.1 mM. to inhibit growth of variants inoculated into 5 mM. xylose or ribose medium[26] suggests that these substrates contain about 100 μg. of glucose which serves as the utilizable carbohydrate.

With the development of protein-free media for the sustained cultivation of mammalian cells,[27,28] amino acid requirements have become potentially useful genetic markers. Intact man, as determined in feeding experiments, requires exogenous isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. In addition to these 8 essential amino acids, HeLa cells require arginine, cysteine, glutamine, histidine, and tyrosine.[29] Some cell strains have been reported to require asparagine, and others require serine in addition to the 13 amino acids necessary for continuous *in vitro* cultivation of HeLa cells. In determining amino acid requirements and using them for genetic analysis, it must be kept in mind that there is a plethora of complicated interactions involving these growth factors. The addition of leucine to a simple, but sufficient medium for *Bacillus anthracis* causes cessation of growth. Ability to multiply is restored upon addition of valine.[30] The most serious complication concerning amino acid requirements, however, is contamination of established cell cultures with pleuropneumonia-like organisms. Over half of mammalian cell lines carried without monitoring for presence of pleuropneumonia-like organisms have been found to be contaminated.[31] Massively infected cell cultures are killed by pleuropneumonia-like organisms and sublines that are chronically infected with low populations of these contaminants grow more slowly than cultures treated with the antibiotic Kanamycin.[32] Most significant, human esophageal epithelial cells infected with pleuropneumonia-like organisms require 1 mM. arginine for maximal growth whereas uninfected cells of the same strain grow optimally with only 0.1 mM. arginine.[33] Clearly, contaminated cell cultures may display nutritional requirements different from sublines free of contaminating microbes.

Differences in specific enzymic activities have been reported for various cell lines. Diverse populations derived from a single clone of mouse cells have been shown to possess dissimilar arginase and β-glucuronidase activities.[22] The disparate phenotypes could be the result of a gene mutation or nuclear reorganization, that is, loss or duplication of some chromosomes. Nonhereditary induction of a previously unexpressed genetic potential, and, conversely, deadaptation resulting in the loss of an enzyme must also be considered. Arginase activity of cells cultivated *in vitro* can be substantially increased by the addition of substrate and ribonucleic acid to the culture.[34] Similarly, alkaline phosphatase can be induced in human cells by the addition of the substrate glycerophosphate to the medium. Moreover, filtrates of medium from induced cell cultures erratically induce alkaline phosphatase activity in unadapted cells.[35] Sterols also markedly affect enzymic activity in mammalian systems;

tryptophan peroxidase activity is stimulated by cortisone, and glutamic-pyruvic transaminase is increased sixfold by treatment with hydrocortisone.[36] In order for a phenotype to be useful in genetic analysis, it must be controlled by structural units that can segregate[37]; nonhereditary physiological states invoked and maintained by novel environmental factors cannot be used for strain identification or genetic analysis.

Derivation of cell lines from different individuals known to possess hereditary, metabolic peculiarities offers the advantage of initial homozygosity for the marker in question. This approach has the disadvantage that it introduces extensive, unmeasurable heterogeneity into the system. Nevertheless, the whole animal seems to provide a more reliable source of hereditary variation than the cell culture bottle. Humans with galactosemia, orotic aciduria, porphyria, acatalasemia, cystathioninuria, pentosuria, alkaptonuria, phenylketonuria, and von Gierke's disease (glucose-6-phosphatase deficient) constitute an available source of cells whose metabolic activities have been partially characterized. It must be emphasized that most cell cultures derived from mammalian tissue have arisen from a special minority of the primary population.[38] Indeed, cell cultures established from day-old rat liver differ antigenically from the tissue of origin.[39] Physiological and antigenic dissimilarity between the tissue of origin and the established cell line isolated from it is not an invariable consequence of tissue culture methodology. Cell cultures prepared from a mouse adrenal tumor have, after several months, retained power to produce tumors in mice and are destroyed by antisera against normal adrenal glands. Moreover, these cell cultures produce steroids in response to stimulation with adrenocorticotropic hormone.[40] Results with cell lines derived from human patients with galactosemia are not as clear-cut, primarily because of the limitations (which are discussed elsewhere in this essay) on determining carbohydrate utilization. Cells of normal origin grow as well in galactose (1 mg./ml.) as in glucose (50 μg./ml.), whereas cells of galactosemic origin do not grow appreciably in galactose medium.[41] The relative contribution of galactose itself and of glucose contaminating the medium to the carbon and energy pool of the cell is not known. Moreover, galactosemic cells may be sensitive to galactose and glucose may serve as a protective factor as well as a substrate.

A final comment on the isolation and maintenance of genetically marked cell cultures is perhaps appropriate. Cross contamination of mammalian cells is now well established[42]; therefore, any presumed variant or recombinant which suddenly differs from the parental lines by many characteristics and which never reverts to the original type should be studied carefully to prove its legitimacy.

LEVELS OF GENETIC INTERACTION

The simplest interaction between two cell populations involves diffusible nutrients. Such a system, based upon the unilateral transfer of inositol synthesized by the mouse fibroblast strain L to inositol-requiring cells such as the

human line HeLa, has been devised.[43] The most direct approach to the alteration of the genetic complement of a mammalian cell is by inserting active genetic material (deoxyribonucleic acid) into appropriately marked, competent recipient cells. It seems reasonably certain that highly polymerized deoxyribonucleic acid can cross the plasma membrane, pass through the cytoplasm, and enter the nucleus.[44] Putative deoxyribonucleic acid has been extracted from bone marrow cells taken from a patient homozygous for the normal gene resulting in the beta polypeptide of hemoglobin A. This nucleic acid has been added to bone marrow cells from a patient with sickle-cell anemia, who is homozygous for a gene resulting in an abnormal beta polypeptide and who makes only abnormal hemoglobin (type S). The treated cells make both hemoglobins A and S.[45] Similar experiments have been carried out, in which ribonucleoprotein brings about alteration of globin synthesis. The power of the nucleoprotein to induce synthesis of new globins was destroyed by trypsin and ribonuclease but not by deoxyribonuclease.[46] The similarity of results, coupled with the inadequate characterization of the nucleic acid in the first mentioned experiments, suggests that these two reports are concerned with the same phenomenon. It is entirely conceivable that informational (messenger) ribonucleic acid or template ribonucleoprotein can enter recipient cells and institute new synthetic processes. Current theory of gene action, however, predicts that such alterations of cellular metabolism will be relatively short-lived. Classically, genetic analyses rely upon fusion of cells, followed by fusion of nuclei. In the fungi, hyphal fusion frequently precedes nuclear fusion by a prolonged interval, thereby giving rise to a persistent heterokaryon. Subsequently, nuclear fusion establishes a rather stable heterozygous diploid, which occasionally gives rise to nuclei with new combinations of genes, either by crossing over during mitosis or by random loss of chromosomes until a haploid nucleus is formed.[47] A possible instance of cell fusion and nuclear fusion, leading to formation of a persistent heterogenomic subline, has been described for mouse fibroblasts. A strain with 55 telocentric chromosomes, one of which was strikingly long, was mixed with a strain possessing 62 chromosomes most of which were telocentric but included 13 metacentrics. A putative hybrid was recovered; its nuclei contained 116 chromosomes, including 1 long chromosome and 9 to 15 metacentrics.[48]

CONCLUDING REMARKS

The major limitation confronting genetic analysis of mammalian cells in continuous culture is not the lack of recombinational mechanisms but the dearth of reliable markers for monitoring these processes. Segregations of whole chromosomes, resulting in hyposomy and polysomy are so regular as to be a nuisance. Ultimately, chromosomal segregation will prove to be a useful tool for assigning genes to linkage groups. Mapping of gene order, however, depends upon gene recombination, and, to date, there is no convincing evidence for crossing over in established, mammalian cell cultures.[37] Now that it is possible to isolate metabolically unique cells from tissue, to perpetuate

established cell lines in defined media, to derive populations from single cells, and even to measure the enzymic activity of single cells,[49] great strides forward in the genetic characterization of cells in continuous culture can be expected at an ever increasing pace.

REFERENCES

1. Delbrück, M., and Stent, G.: On the Mechanism of DNA Replication. In McElroy, W., and Glass, B. (Eds.): *The Chemical Basis of Heredity*. Baltimore: The Johns Hopkins Press, 1957, pp. 699–736.
2. Spiegelman, S.: The Relation of Informational RNA to DNA. Cold Spring Harbor Sympos. Quant. Biol. *26*:75–90, 1961.
3. Crick, F.; Barnett, L.; Brenner, S.; and Watts-Tobin, R.: General Nature of the Genetic Code for Proteins. Nature *192*:1227–1232, 1961.
4. Lengyel, P.; Speyer, J.; Basilio, C.; and Ochoa, S.: Synthetic Polynucleotides and the Amino Acid Code, III. Proc. Nat. Acad. Sc. *48*:282–284, 1962.
5. Ephrussi, B.: *Nucleo-cytoplasmic Relations in Micro-organisms*. Oxford, England: The University Press, 1953.
6. Hartman, P., and Goodgal, S.: Bacterial Genetics (With Particular Reference to Genetic Transfer). Annual Rev. Microb. *13*:465–504, 1959.
7. Chang, R. S., and Geyer, R.: Propagation of Conjunctival and HeLa Cells in Various Carbohydrate Media. Proc. Soc. Exp. Biol. *96*:336–340, 1957.
8. Pace, D., and Lefkos, A.: Effects of Cell Density of Cell Growth in a Clone of Mouse Liver Cells. J. Nat. Cancer Inst. *19*:1065–1075, 1957.
9. Cooney, W., and Bradley, S. G.: The Action of Cycloheximide on Animal Cells. Antimicrob. Agents & Chemother.—1961, pp. 237–244, 1962.
10. Cohn, M., and Horibata, K.: Physiology of the Inhibition by Glucose of the Induced Synthesis of the β-galactoside-Enzyme System of *Escherichia coli*. J. Bact. *78*:624–635, 1959.
11. Hsu, T.; Billen, D.; and Levan, A.: Mammalian Chromosomes *in vitro;* XV. Patterns of Transformation. J. Nat. Cancer Inst. *27*:515–541, 1961.
12. Clausen, J., and Syverton, J.: Comparative Chromosomal Study of 31 Cultured Mammalian Cell Lines. J. Nat. Cancer Inst. *28*:117–147, 1962.
13. Stern, C.: The Chromosomes of Man. J. M. Educ. *34*:301–314, 1959.
14. The Human Chromosome Study Group: A Proposed Standard System of Nomenclature of Human Mitotic Chromosomes. J. Heredity *51*:214–221, 1960.
15. Stern, C.: Genic Action as Studied by Means of the Effects of Different Doses and Combinations of Alleles. Genetics *28*:441–475, 1943.
16. Sturtevant, A. H.: The Effects of Unequal Crossing Over at the Bar Locus in Drosophila. Genetics *10*:117–147, 1925.
17. Johannsen, W.: *Elemente der Exakten Erblichkeitslehre mit Grundzügen der Biologischen Variationsstatistik*. Jena: Verlag von Gustav Fischer, 1913.
18. Bradley, S. G., and Syverton, J.: Inheritance of Capability of Human Esophageal Epithelial Cells to Grow with Diverse Carbohydrates. Proc. Soc. Exp. Biol. *103*:215–221, 1960.
19. Chang, R. S.: Isolation of Nutritional Variants from Conjunctival and HeLa Cells. Proc. Soc. Exp. Biol. *96*:818–820, 1957.
20. Katsuta, H.; Takaoka, T.; and Kikuchi, K.: Further Studies on Amino Acid Requirements of Strain L Cells (Mouse Fibroblast) in Synthetic Media. Jap. J. Exp. M. *31*:125–136, 1961.
21. Hsu, T., and Somers, C.: Establishment and Properties of L Cells Resistant to Bromodeoxyuridine. Abstracts of Papers Presented at the First Annual Meeting of the Am. Soc. Cell Biol., p. 94, 1961.
22. Sanford, K.: Alterations in Morphology, Arginase and β-glucuronidase Within a Clone of Mouse Tumor Cells *in vitro*. J. Nat. Cancer Inst. *26*:1193–1219, 1961.
23. Bailey, J.; Gey, G.; and Gey, M.: The Carbohydrate Nutrition and Metabolism of a Strain of Mammalian Cells (MB III Strain of Mouse Lymphoblasts) Growing *in vitro*. J. Biol. Chem. *234*:1042–1047, 1959.

24. Bradley, S. G., and Syverton, J.: Characterization of a Nutritional Variant of Human Esophageal Epithelial Cells. Exp. Cell Res. *27*:25–30, 1962.
25. Swim, H. E.: Microbiological Aspects of Tissue Culture. Annual Rev. Microb. *13*:141–176, 1959.
26. Chang, R. S.: Genetic Study of Human Cells *in vitro;* Carbohydrate Variants from Cultures of HeLa and Conjunctival Cells. J. Exp. M. *111*:235–254, 1960.
27. McQuilkin, W.; Evans, V.; and Earle, W.: The Adaptation of Additional Lines of NCTC Clone 929 (Strain L) Cells to Chemically Defined Protein-free Medium NCTC 109. J. Nat. Cancer Inst. *19*:885–907, 1957.
28. Eagle, H.: The Sustained Growth of Human and Animal Cells in a Protein-free Environment. Proc. Nat. Acad. Sc. *46*:427–432, 1960.
29. Eagle, H.: The Specific Amino Acid Requirements of a Human Carcinoma Cell (Strain HeLa) in Tissue Culture. J. Exp. M. *102*:37–48, 1955.
30. Gladstone, G.: Inter-relationships Between Amino Acids in the Nutrition of *B. anthracis*. Brit. J. Exp. Path. *20*:189–200, 1939.
31. Pollock, M.; Kenny, G.; and Syverton, J.: Isolation and Elimination of Pleuropneumonia-like Organisms from Mammalian Cell Cultures. Proc. Soc. Exp. Biol. *105*:10–15, 1960.
32. Fogh, J., and Hacker, C.: Elimination of Pleuropneumonia-like Organisms from Cell Cultures. Exp. Cell Res. *21*:242–244, 1960.
33. Kenny, G.: The Effect of Pleuropneumonia-like Organisms (Mycoplasma) on Growth of Mammalian Cells *in vitro*. Ph.D. Thesis, University of Minnesota, Minneapolis, 1961.
34. Klein, E.: Studies on the Substrate-induced Arginase Synthesis in Animal Cell Strains Cultured *in vitro*. Exp. Cell Res. *22*:226–232, 1961.
35. Cox, R., and Pontecorvo, G.: Induction of Alkaline Phosphatase by Substrates in Established Cultures of Cells from Individual Human Donors. Proc. Nat. Acad. Sc. *47*:839–845, 1961.
36. Najjar, V. A.: General Considerations in Activity and Induction of Enzymes. In Fomon, S. J. (Ed.): *Disturbances of Cellular Metabolism in Infancy,* Report of the Thirty-second Ross Conference on Pediatric Research. Columbus, Ohio: Ross Laboratories, 1959, pp. 15–18.
37. Pontecorvo, G.: Methods of Microbial Genetics in an Approach to Human Genetics. Brit. M. Bull. *18*:81–84, 1962.
38. Zaroff, L.; Sato, G.; and Mills, S.: Single Cell Platings from Freshly Isolated Mammalian Tissue. Exp. Cell Res. *23*:565–575, 1961.
39. Sato, G.; Zaroff, L.; and Mills, S.: Tissue Culture Populations and Their Relation to the Tissue of Origin. Proc. Nat. Acad. Sc. *46*:963–972, 1960.
40. Sato, G., and Buonassisi, V.: The Culture of Differentiated Tumors. Abstracts of Papers Presented at the First Annual Meeting of the Am. Soc. Cell Biol., p. 189, 1961.
41. Krooth, R., and Weinberg, A.: Studies on Cell Lines Developed from the Tissues of Patients with Galactosemia. J. Exp. M. *113*:1155–1171, 1961.
42. Brand, K. G., and Syverton, J.: Results of Species-specific Hemagglutination Tests on "Transformed," Nontransformed, and Primary Cell Cultures. J. Nat. Cancer Inst. *28*:147–157, 1962.
43. Eagle, H.; Agranoff, B.; and Snell, E.: The Biosynthesis of Meso-Inositol by Cultured Mammalian Cells, and the Parabiotic Growth of Inositol-dependent and Inositol-independent Strains. J. Biol. Chem. *235*:1891–1893, 1961.
44. Borenfreund, E., and Bendich, A.: A Study of the Penetration of Mammalian Cells by Deoxyribonucleic Acids. J. Biophys. Cytol. *9*:81–91, 1961.
45. Kraus, L.: Formation of Different Haemoglobins in Tissue Culture of Human Bone Marrow Treated with Human Deoxyribonucleic Acid. Nature *192*:1055–1057, 1961.
46. Weisberger, A. S.: Induction of Altered Globin Synthesis in Human Immature Erythrocytes Incubated with Ribonucleoprotein. Proc. Nat. Acad. Sc. *48*:68–80, 1962.
47. Bradley, S. G.: Parasexual Phenomena in Microorganisms. Annual Rev. Microb., 1962 (in press).
48. Barski, G.; Sorieul, S.; and Cornefert, F.: "Hybrid" Type Cells in Combined Cultures of Two Different Mammalian Cell Strains. J. Nat. Cancer Inst. *26*:1269–1291, 1961.
49. Rotman, B.: Measurement of Activity of Single Molecules of β-D-galactosidase. Proc. Nat. Acad. Sc. *47*:1981–1991, 1961.

Virus Susceptibility of Mammalian Cells

Joseph L. Melnick, Ph.D.

IN the earliest phase of their study it was soon realized that viruses are very selective in the types of cells which they attack. Rabies virus will infect and produce fatal disease in every mammalian species in which it has been tested. In contrast, infectious hepatitis, so far at least, has been limited to growth only in the human species.

It is well established that a virus can infect certain types of cells growing *in vitro,* but not others, and that the susceptibility or the resistance of the cells is a property which is inheritable from one generation to the next. In regard to viral susceptibility, animal cells are similar to bacteria that have a genetically controlled susceptibility specific for certain bacterial viruses.

In this paper I shall not consider the role of virus-induced resistance, as mediated through interferon or through antibodies.

CELL RECEPTORS AS A SUSCEPTIBILITY FACTOR

Viral susceptibility of bacteria has been well studied. Receptor sites must be present on the cell surface for the invading bacteriophage to attach successfully to its host cell. Close variants of the same bacterial species may lack these receptors and they are completely resistant to infection by the bacteriophage. Although a great deal about cell receptors has been learned in the past decade, we are still far from a clear understanding of why animal viruses in particular have predilections only for certain cells. A virus which will attack certain types of cells *in vitro* may have no effect on the cells of this tissue *in vivo.* Monkey testicular and kidney cells are refractory to infection *in vivo,* yet when they are cultured *in vitro* the cells from these same tissues become fully susceptible to the virus[1,2] even when taken from a hyperimmunized animal.[3] It was recently found that all poliovirus-susceptible cells *in vitro* contain receptor material which is able to bind virus when the virus is incubated with cell homogenates (particularly the microsome fraction), whereas all resistant cells tested lack such receptor activity.[4] In this case the susceptible cells were of primate origin and the resistant cells of rodent origin. This lack of receptor seems to be the main reason for the poliovirus resistance of nonprimate cells, for such nonprimate cells can support the replication of poliovirus if the infection is initiated with viral nucleic acid. Under certain experimental conditions, viral nucleic acid, free of its protein coat, can bypass the receptors and enter the cell.[5,6]

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Recently Holland[7] followed up an early observation which Thomas Francis made here in Ann Arbor[8] that poliovirus can be bound by specific substance in brain tissue of primates. Holland has shown that poliovirus susceptibility of non-neural human tissue in cell culture is due to the *in vitro* development of receptor material which is not present in cells growing *in vivo*. Even the conditions under which cells are kept *in vitro* greatly influence whether or not receptor substance appears. For example, when human amnion cells were left on the amniotic membrane they were resistant to poliovirus and contained no receptor substance. However, when these cells were removed from the membrane by trypsinization and cultivated directly on the glass they then produced receptor substance and became susceptible to poliovirus within a week. In contrast, when intact amniotic membranes were cultivated *in vitro* with the cells in normal contact with the collagen membrane they remained resistant to poliovirus and also failed to produce receptor substance.

Thus Holland concluded that "enterovirus tropisms are due to a large extent to affinity or lack of affinity between virus protein surfaces and a specific component of the surfaces of the various cells of the body. The wide range of enterovirus susceptibility of most human cells after *in vitro* cultivation appears to be a consequence of receptor synthesis *in vitro*—a function which is normally repressed in most cells in their differentiated state in the body."[7]

ADSORPTION AND EARLY FATE OF VIRUS IN SUSCEPTIBLE CELLS

The experiments mentioned above show that an animal virus, as in the case of bacteriophage, may also be dependent upon cell receptors if it is to initiate an infection. Using poliovirus labeled with P^{32}, Joklik and Darnell[9] were able to follow the adsorption and early fate of the virus in susceptible HeLa cells. Virtually all the virus particles attach to susceptible cells. Once adsorbed, the fate of the virus particles varies. Over 50 percent of the particles elute from the cells. Such virus particles are different from the original ones, for they now are unable to adsorb and to initiate infection. However, functional infective RNA can be extracted from such damaged virus particles. It is noteworthy that the rate and extent of elution are independent of the multiplicity of infection, even with a range of virus to cell multiplicity varying from 1 to 1,000. Also, the rate and extent of elution are independent of the cell concentration during either the adsorption period or the elution period. When the cells were examined at the end of the elution period, they appeared to be in excellent condition and their counts were identical to those at the start. Thus, elution of virus cannot be explained by the fragmentation of some cells which have adsorbed the virus. The virus which elutes is extremely uniform in this respect. Even the final 10 percent of the virus which will eventually elute does so as rapidly as the first 10 percent.

Joklik and Darnell[9] found that almost all the adsorbed virus which does not elute is rapidly broken down within the cell and appears in acid-soluble material. It is well known in poliovirus infections that cells produce a large excess of physical viral particles in relation to infectious particles. It would

appear from the study just cited that all virus particles are capable of adsorbing to cells, and thus they contain viral protein in a functional form. Also, eluted virus contains as much infectious RNA as particles which have never been exposed to susceptible cells. The authors believe that it is a chance phenomenon whether or not particles are inactivated and eluted and others enter the cell and initiate infection, and that the ability to cause infection is not restricted to a small fraction of the total virus population.

Early in the breakdown of intracellular virus there is a conversion of the virus to a state in which it is susceptible to RNAase. The mechanism of this step or the extent of removal of the protein coat is not known. However, it all takes place relatively quickly, for it is 50 percent completed within 15 to 20 minutes after virus adsorption and is largely complete within the first hour after infection. The next hour or two is a true eclipse period, for the formation of viral RNA and protein does not begin until at least two and one-half hours after infection.

One of the most exciting recent observations in virology has been that infectious viral nucleic acid free of protein coat can infect cells that are resistant to infection with the complete virus particle.[5,6] For example, after exposure to RNA isolated from poliovirus, the normally resistant cells from rabbit, mouse, guinea pig, hamster, or chick yield complete poliovirus. The new virus is precisely the same as the original poliovirus which yielded the nucleic acid, in that it also cannot infect rodent cells but can infect primate cells. Thus, all the data presented strengthen the widely held view that the protein coat, or capsid, of viruses does not function merely as a protective mechanism for the nucleic acid, but plays an important role in determining which cells are susceptible to the virus. The protein coat provides specific means for the efficient attachment to and penetration of the cells in which the virus can replicate. Since viral nucleic acid by itself lacks the antigenic component imparted by the protein capsid, it cannot be inactivated by specific antibodies. Thus the possibility has been raised by several that viral nucleic acids may be one means by which viral infections may continue to smolder even in the presence of circulating antibodies. To curtail this phase of viral infection one would have to call upon the enzymes, the nucleases, which are present in sera to a varying degree.

POSTADSORPTION BLOCK

This type of resistance is not due to the absence of viral receptors on the cell surface. Cell cultures from the South American capuchin monkey support only a limited multiplication of one enterovirus,[2,10] while those of the African red grass monkey (*Erythrocebus patas*) show similar low susceptibility to other enteroviruses[11]; yet the blocks are located at different points in the infectious cycle.

In studies on capuchin monkey cells it was found that these cells fail to adsorb significant amounts of poliovirus (enterovirus 1), and that the limited multiplication of this enterovirus in such cultures which showed no cytopathic

changes was explained by the fact that only 1 in 10,000 cells becomes infected.[10] The situation with enteroviruses 12 and 33 (Coxsackie A9, ECHO 1) in patas cells was different, in that these viruses adsorbed as well on both patas and rhesus, although plaques appeared only in the fully susceptible rhesus cultures. Thus the failure to induce plaque formation may be due to the failure to adsorb, as with poliovirus in capuchin cells, or may be due to other blocks in the infection cycle. Patas cells in fluid media supported the growth of enteroviruses 12 and 33 to a limited degree. Although the time of virus formation was the same as in rhesus, the yield in patas was only about 3 percent of that in rhesus.[11]

These two monkey species are genetically quite different. Patas cells have a chromosome count of 54, and rhesus one of 42. That genetic factors may be responsible for inhibiting viral multiplication has also been found by Sabin[12] in his *in vivo* study of the 17-D strain of yellow fever and other group B arbor viruses in PRI mice. Although 17-D virus multiplied in the brains of the resistant PRI mice, it produced no disease in them as it did in Swiss mice. The peak titers in resistant mice were only a small fraction of those achieved in the brains of susceptible Swiss mice. This is similar to the lack of cytopathogenicity of enteroviruses 12 and 33 for patas cells, even though these cells supported a limited multiplication of these viruses.

Cell variants of a single line, in this case HeLa, have also been obtained and show differences in their susceptibility to poliovirus. For example, Vogt and Dulbecco,[13] by exposing a cell culture to poliovirus and then curing the infection in surviving cells with antiserum, obtained a variant which adsorbed virus equally as well as the parent cell line and even had the same latent period. However, there was such a low efficiency of infection after adsorption that the over-all susceptibility of the variant cell line was only about 7 percent that of the parent. This type of relative resistance, in which cells adsorb virus equally well but only one genetic variant produces virus to high efficiency, is due to an inherited property of the cells which decreases the possibility that adsorbed virus will enter the cell and multiply in competent fashion. It is different from the resistance due to lack of receptor substances on the cell surface.

The Vogt and Dulbecco resistant line was composed of fibroblastic cells. By cloning HeLa cells, Darnell and Sawyer[14,15] obtained epithelial sublines which showed the same type of postadsorption block. However, they found that while the cell lines differed fifteenfold in their susceptibility to whole virus, they were equally susceptible to free viral nucleic acid. It would appear that the block was caused by a failure of the infected cells to deproteinize the whole virus and liberate the free nucleic acid within the cell.

Postadsorption blocks may come later in the infectious cycle, as shown with the use of inhibitors.[16–18] When p-fluorophenylalanine—a poliovirus inhibitor discovered here in Ann Arbor—is used at concentrations of 100 μg./ml., synthesis of both viral RNA and viral protein is completely suppressed. At low concentration of 4 μg./ml. (0.025 mM.), the synthesis of infectious RNA

proceeds on schedule but no whole functional virus is made. Perhaps the virus protein coat is not made or it is made inadequately, or its combination with the nucleic acid is blocked. We do know from experiments with vital dyes[19] that these basic compounds can become attached to viral nucleic acid in the cell, but nevertheless the protein coat is then added to complete the maturation process. In the process, such virus becomes sensitive to inactivation by light.[20] Postadsorption blocks of the type produced by chemical inhibitors have not been observed as yet in naturally resistant cells.

ENVIRONMENTAL FACTORS

Nutritional factors which influence the degree of susceptibility of cells to poliovirus have been discussed recently.[21] I would like to mention some observations with ordinary nutrients which are not usually considered to be important factors.

Glycine is required for the growth of monkey kidney cells. However, even when present in concentrations lower than that required by the cells for growth, this amino acid significantly inhibits the synthesis of poliovirus.[22] Studies of the first cycle of virus synthesis, where the glycine has been added after adsorption, indicate that 3×10^{-4} molar glycine decreases the yield of virus about 90 percent and prolongs the eclipse period by about one hour. Figure 1 shows that in the presence of glycine, plaque numbers are reduced

Fig. 1.—Glycine inhibition of poliovirus plaques on monkey kidney cells. The same number of virus particles was seeded on each monolayer. From left to right: (1) absence of glycine, (2) inhibition at 2×10^{-3} M glycine, (3 and 4) less inhibition with decreasing molarities of glycine.

Fig. 2.—Effect of glucose and levulose on ECHO-1 plaques on monkey kidney cells. Left to right: (1) ECHO-1 plaques on control monkey kidney cells grown on glucose, plus glucose in overlay, (2) cell resistance increased by substituting levulose for glucose in overlay, (3) cell resistance of levulose-grown cells overcome by substituting glucose for levulose in overlay, (4) cell resistance in cells grown on levulose, plus levulose in overlay.

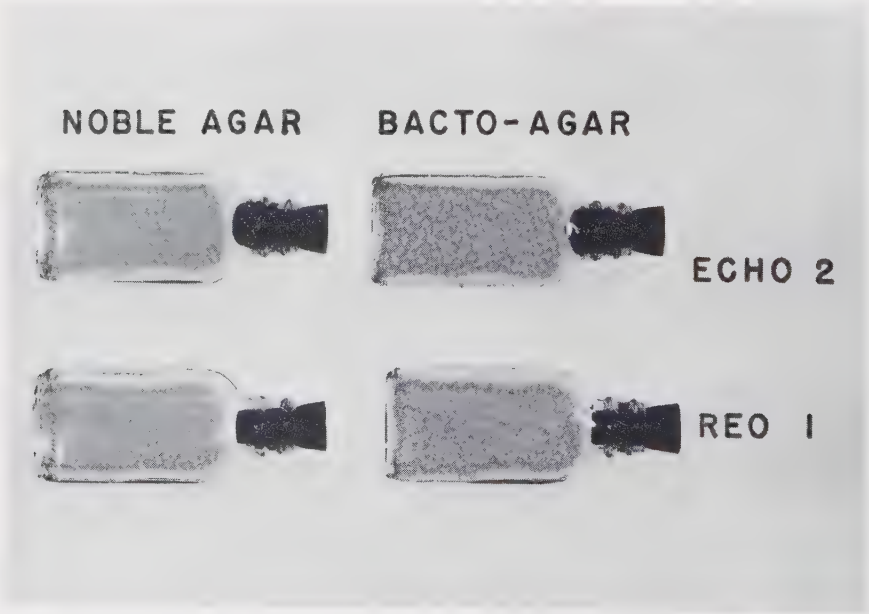

Fig. 3.—Cells under less purified bacto-agar overlay (cultures on right) are highly susceptible to ECHO-2 and reo-1 viruses in contrast to cells under Noble agar (cultures on left).

under a protein-free medium used in the agar overlay, and the rate of growth of the plaques is retarded.

Within the enterovirus types, substitution of one hexose by another may change a susceptible cell into a resistant one. Substitution of levulose for glucose in the growth medium has been shown to yield monkey kidney cells with lower susceptibility to certain enteroviruses (see Figure 2).[23]

Even the type of agar used in the overlay may play a crucial role. Under Noble agar, certain ECHO viruses (types 2, 3, 5, 6) have failed to grow.[24] An inhibitor has been removed by treatment with DEAE dextran[25] or simply by replacement with a "less-purified" agar[26] (see Figure 3), so that plaques can now be produced readily with these viruses.

Variations in the concentration of cations in the nutrient medium are also important. As shown in Figures 4 and 5, increasing the concentration of Mg ions to 25 mM. enhances the susceptibility of monkey kidney cells to poliovirus and certain other enteroviruses, apparently by causing an earlier release of virus from infected cells.[27]

In the course of studies on virulent and attenuated polioviruses, certain genetic markers were found which could be detected by differences in cell

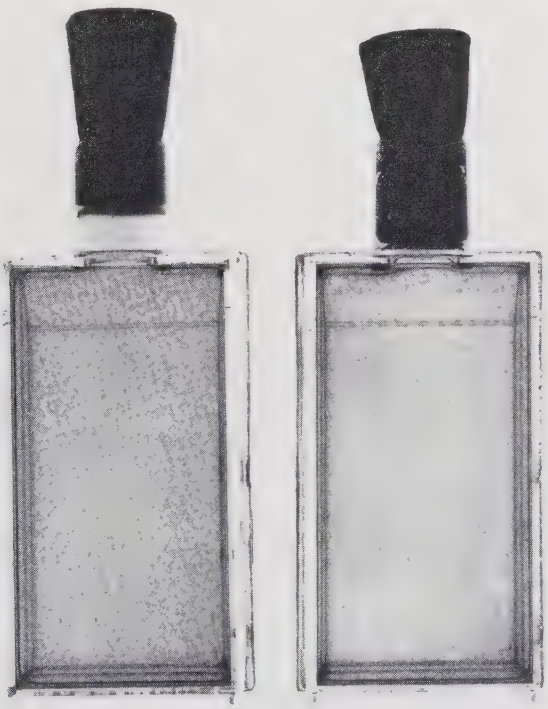

Fig. 4.—Monkey kidney cells under overlay containing 25 mM Mg^{++}. Right-hand bottle shows increased cell susceptibility to poliovirus (LSc) when compared with cells under 1 mM Mg^{++} overlay (left). Photographed on second day after seeding.

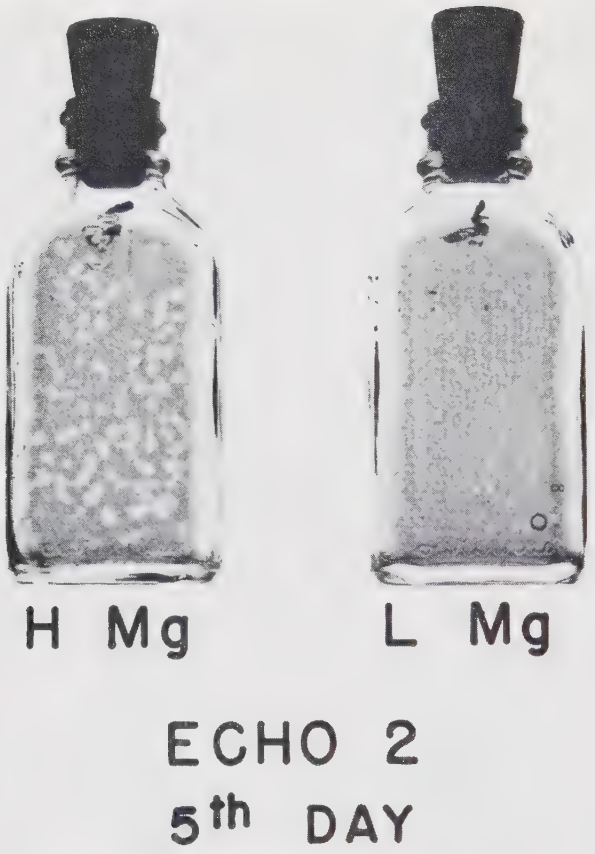

Fig. 5.—Growth of ECHO-2 virus on monkey kidney cells in media containing high (25 mM) and low (1 mM) Mg^{++} concentrations, 5 days after seeding.

susceptibilities to these viral strains. These differences could be brought out by varying the conditions under which the monkey kidney cells were kept. Changing the cell environment does not influence the cell susceptibility to virulent but only to attenuated strains. Thus, decreasing the bicarbonate concentration of the medium favored the growth of d+ strains (see Figure 6).[28] Increasing the temperature of the cultures to 40° C. favored the growth of T+ (rct/40$^+$) strains.[29]

Another useful marker has been the fact that the MS cell line is highly susceptible to virulent but not to attenuated poliovirus strains.[34] Recently McBride[30] has shown that this marker is associated with a cystine requirement of attenuated strains. If cysteine (0.2 mg./ml.) is added to MS cells they become as susceptible to attenuated strains as they are to virulent strains.

We have been interested in the correlation of the above *in vitro* markers of poliovirus and their *in vivo* neurovirulence.[31] Recently our laboratory has found another marker which seems to be associated with neurovirulence—

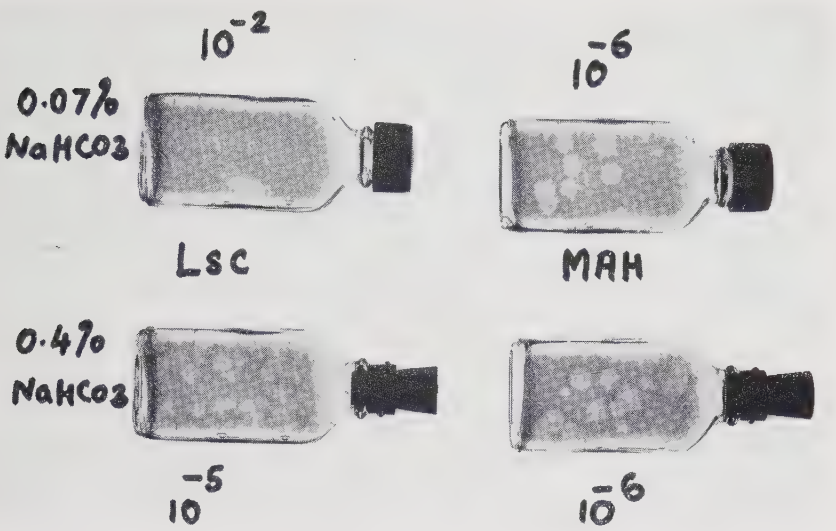

Fig. 6.—Resistance of cells to attenuated poliovirus type 1 (LSc) is enhanced by decreasing $NaHCO_3$ concentration in overlay (cultures on left). Growth of virulent type 1 (Mahoney) is unaffected by altering $NaHCO_3$ concentration (cultures on right).

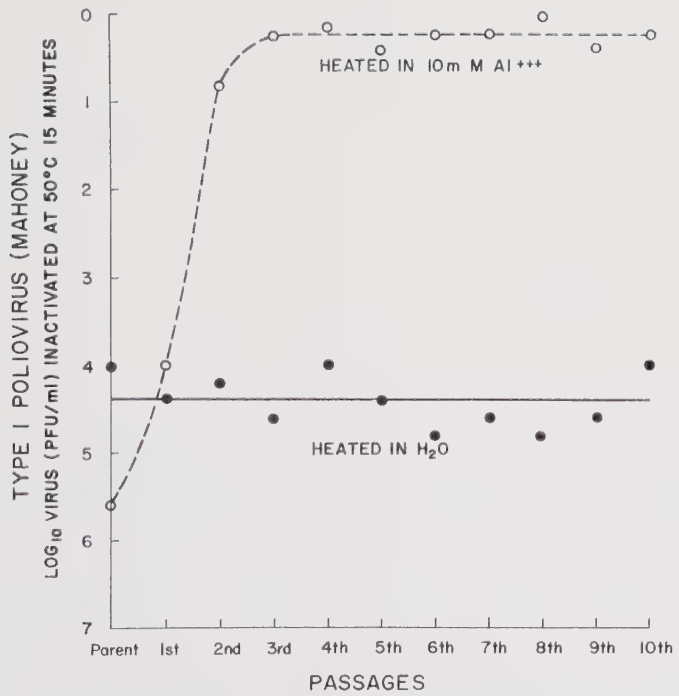

Fig. 7.—Selection of heat resistant Mahoney progeny. White circles indicate survivors of heat treatment in 10 mM Al^{+++} after each of 10 passages. Black circles indicate survivors after heat inactivation *in water* remained consistently low for 10 passages.

one which does not depend upon changing the cell environment. Virulent but not attenuated strains are susceptible to inactivation if heated at 50° C. for 15 minutes in the presence of low (10 mM.) concentrations of Al ions.[32] By selecting the few progeny of virulent strains which resist the Al-heat treatment and passing them, and re-subjecting them to the heat treatment, we have been able after several passages to change the character of the virus so that it becomes as resistant as an attenuated strain (see Figure 7).[26] A comparison at different Al^{+++} concentrations of the parent virulent Mahoney virus with the tenth Al-heat passage progeny and with attenuated LSc virus is shown in Figure 8. No differences can be seen between the tenth passage material and

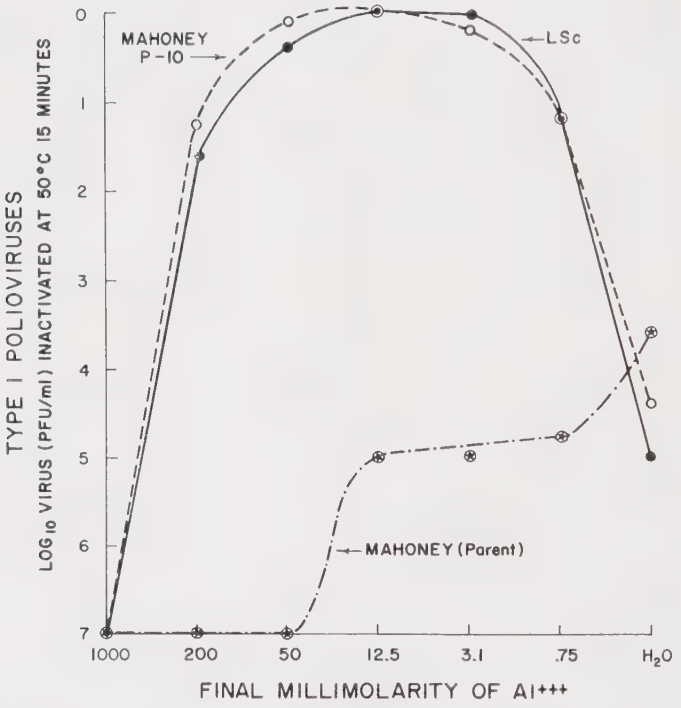

Fig. 8.—After 10 treatments and passages, virulent virus behaves like an attenuated strain. White circles and broken line indicate survivors of 10th passage Mahoney progeny to heat treatment in different concentrations of Al^{+++}. Black circles and solid line indicate similar results with parent attenuated LSc after the same treatment. Starred circles and dot-dash line represent survivors of Mahoney parent strain, and indicate the lability of the virulent parent strain.

the attenuated LSc strain. We then tested these strains for the d and T markers and also for intracerebral neurovirulence in monkeys. The results are listed in Table 1.

After 3 passages, the virus was still d+ and T+ but only half the monkeys succumbed. After 10 passages the virus had lost its intracerebral neurovirulence for monkeys. Controls passed simultaneously in $AlCl_3$ but not

heated, or progeny recovered from 10 serial passages using the same heat treatment in water without Al ions present, remained virulent. Of further interest was the finding that the newly attenuated Mahoney virus retained its d+ marker. This confirms several other observations indicating that the d locus is not related to virulence even though it is usually present in virulent strains.

TABLE 1.—*Variations in Cell Susceptibility to Type 1 Poliovirus*

Strain	d	T	A*	Monkey Intracerebral Neurovirulence
Original Mahoney	+	+	+	8/8
3rd passage Al, heat	+	+	±	3/6
10th passage Al, heat	+	—	—	0/8
10th passage Al, no heat	+	+	+	3/4
10th passage H_2O, heat	+	+	+	2/2

* Susceptible to heating at 50° C. for 15 minutes in 10 mM $AlCl_3$.

I have brought these data to your attention to show how changes in cell susceptibility both *in vitro* and *in vivo* are being employed for studying genetic changes of the virus itself. Starting with an A+ virus, we find that monkey kidney cells *in vitro* are equally susceptible to it at 37° and at 40° C., and *in vivo* the monkey CNS is susceptible, even when fever elevates the temperature to 40° C. With the selection of an A mutant, we now find that monkey kidney cells have lost their susceptibility to the mutant at 40° C., and monkeys are no longer susceptible to the new virus.

FAILURE OF ENTEROVIRUS CLASSIFICATION SYSTEM BASED ON CELL SUSCEPTIBILITY

A practical demonstration of the variability in cell susceptibility may be seen in the recent decision of the Enterovirus Committee to abandon the old subgrouping of these viruses.[42] As shown in Table 2 polio, Coxsackie, and ECHO viruses were originally placed in subgroups because of the susceptibilities of the cell cultures *in vitro* or of the tissues *in vivo*. In almost every instance, however, exceptions in cell susceptibility were found, either as more virus strains were isolated and studied or as established strains were manipulated in the laboratory and tested for such differences. Some of these exceptions are noted in the footnotes for Table 2.

In view of this situation the Enterovirus Committee has recently suggested that the cell or host susceptibility no longer be used to subgroup these viruses, and a simple numbering system has been proposed to include all members of the group.[42]

At least 59 members are known in the enterovirus group (see Table 3). In the future as their properties become further recognized, it is contemplated that subgroups will be found. Viral properties more stable than cell suscepti-

TABLE 2.—*Enteroviruses: Growth in Monkey Kidney and Human Cell Cultures, and Pathogenicity for Animals*

STRAIN	MONKEY KIDNEY	HELA AND OTHER HUMAN CELLS	PATHOGENICITY	
			Mice	Monkeys
Polio 1–3	+	+	o[a]	+[b]
Coxsackie A	o[c]	o[d]	+[c]	o[e]
Coxsackie B	+	+	+[c]	o[e]
ECHO	+	o[d]	o[f]	o[e]

[a] Some strains of each type have been adapted to mice.

[b] Attenuated strains used for oral vaccine produce mild localized lesions when inoculated intraspinally and almost no lesions when inoculated intracerebrally.

[c] Coxsackie A9 and B strains grow readily in monkey kidney cells; some strains grow poorly in mice and fail to produce disease in them.

[d] Some strains grow preferentially in, or have been adapted to, human cell cultures.

[e] Coxsackie A7 produces a severe polioencephalomyelitis in monkeys; other Coxsackie and ECHO strains produce *mild* lesions in the CNS resembling mild poliomyelitis.

[f] While the prototype and other strains of ECHO 23 are not pathogenic for mice, a number of other strains, especially after passage in monkey kidney cells, produce paralysis in mice (severe Coxsackie type myositis).

TABLE 3.—*Members of the Enterovirus Group and Corresponding Enterovirus Numbers*

Virus	Enterovirus Number
Polio 1–3	1–3
Coxsackie A 1–24	4–26
Coxsackie B 1–6	27–32
ECHO 1–28	33–58
....	59
ECHO 1, ECHO 8	33
ECHO 9, Coxsackie A 23	40

bility (*in vivo* or *in vitro*) should be the basis for the future subgrouping. By stable properties we mean size and fine structure of the virus, type of nucleic acid, presence of essential lipids, growth in the cytoplasm or nucleus, and other characteristics of this nature.

CELL TRANSFORMATION BY INFECTING VIRUS

Studies on the conversion of cells from one type to another under the influence of an infecting virus are under active investigation in a number of laboratories working on tumor viruses, especially polyoma and Rous sarcoma viruses. In their recent study on avian myeloblastosis virus (AMV) Baluda and Goetz[33] have demonstrated the importance of susceptible target cells being present for conversion to take place. If target cells are absent in a culture exposed to virus the culture becomes infected and produces virus, but the cells do not transform to neoplastic types. The target cells which are converted by the virus may be the normal precursors of the myeloblasts and osteoblasts.

Cultures of cells from different embryonic and adult organs show marked differences in their susceptibility to conversion by AMV. Cell cultures from the muscle, liver, and brain of 5 to 7 day old embryos failed to convert when up to $10^{8.5}$ infectious doses were added. However, spleen cell cultures from 16 day old embryos are readily converted even by 1 infective dose of virus. At this stage of embryonic development, the spleen has become hematopoietic.

Variations in cell susceptibility to conversion are not due to differences in adsorption of virus, or to differences in the degree to which the virus multiplies. Virus infects and multiplies in cells which fail to convert as readily as it does in those which do.

Cultures from the same tissue contain different types of cells, not all of which can undergo conversion.[33] Of colonies obtained from cloned cells, conversion appeared as follows:

none for kidney cells of 10 day old embryos,
5 percent for kidney cells of 16 day old embryos,
80 percent for spleen cells of 17 day old embryos,
none for spleen cells of 109 day old chicken.

Cells which attach most readily to glass—even from spleen—are not converted as are those which attach poorly. Without the virus as a transforming agent, there would be no way of distinguishing one cell type from another in these cultures. I would expect that this sort of approach to the genetic constitution of cells of the same animal, or even of the same tissue or of the same culture, is one which will be receiving increasing attention.

VIRAL SUSCEPTIBILITY OF SO-CALLED "TRANSFORMED" CELLS

The specificity of viral susceptibility has proved useful in following the genetics of so-called transformed or altered cells, that is, cells which in the absence of added virus have suddenly changed in culture and almost always have proved capable of continuous propagation. In addition, altered cells differ from those of primary explants in their morphology. Some work that we were involved in a few years ago is illustrative of the way in which viruses can help sort out the origin of such so-called transformed cells. Westwood et al.[35] observed a small plaque of epithelial cells in a degenerating culture of fibroblasts obtained from embryo rabbit kidney. A serial line of epithelial cells was grown from this culture and found to be susceptible to poliovirus.[36] Because of the many advantages of having a nonprimate cell line for growing poliovirus, this line was welcomed by workers throughout the world. By means of a conventional complement fixation test we soon found that this line of rabbit kidney, the so-called ERK line, had a strong antigenic overlap with HeLa cells, but not with rabbit kidney.[37] This suggested to us that HeLa, or a similar cell, contaminated the embryonic rabbit cultures and that it was this primate cell which outgrew the cells in the original culture and was being propagated in series. This would be a straightforward explanation of why the transformed line now supported the growth of poliovirus. Subsequent investigations in a number of laboratories using more elegant antigenic tests or

chromosome analysis led to the same conclusion.[38-40] In the course of carrying out these tests, we studied one cell line sent to us by Parker of Toronto. He had obtained an altered cell from monkey kidney cell cultures which he had found to be resistant to poliovirus. We soon found that this cell line did not possess primate complement-fixing antigen. Subsequent work by the group in Toronto[38] showed that the altered monkey lines which had become resistant to poliovirus no longer had the chromosome complement of the monkey but rather possessed that of the mouse, and contained mouse antigen.

Results from a number of laboratories now suggest that transformed cell lines which change their viral susceptibility may often arise not by cell alterations or transformations, but by contamination of normal cultures with other cell lines of the primate type such as HeLa or of the mouse type such as the L strain. Such cells are capable of indefinite propagation in series and have chromosome characteristics different from those of the cells of their supposed

TABLE 4.—*Cell Susceptibility of Primary and Transformed Cells*

Type of Tissue	Susceptibility to Poliovirus	Species Antigens and Chromosomes
Rabbit kidney, primary	—	Rabbit
Rabbit kidney, transformed	+	Human
Monkey kidney, primary	+	Monkey
Monkey kidney, transformed A	—	Mouse
Monkey kidney, transformed B	+	Human

Some lines of Cercopithecus monkey kidney cells are known which retain full susceptibility to vacuolating and polioviruses.

origin. Table 4 shows the correlation between viral susceptibility and other analyses of the genetic constitution of the cell strain. Of course other cell lines have been developed, as, for example, the ones by Barski et al.,[41] from human or rabbit origin. After two years of cultivation *in vitro,* these workers showed that the cells not only retained their immunological characteristics, but also retained the same viral susceptibilities as the parent cells.

SUMMARY

Viruses are becoming useful for probing into the genetic material of animal cells as they have for bacterial cells. However, a number of factors must be taken into account as we develop our understanding of the virus-cell reaction. We have seen that at its simplest level it is not difficult to say whether or not a cell population differs from others by its susceptibility to a virus. We have also seen that if small changes are made in the environment of a cell—such as substituting fructose for glucose, varying the temperature, or changing the concentration of glycine, cysteine, bicarbonate, or Mg ions—each change by itself is enough to alter the susceptibility of the cell to a virus. We have also seen that when a virus is altered genetically the susceptibility of cells to it may be greatly altered.

Some of the activities that occur in infected cells have been discussed. Cell resistance can be due to a variety of factors: lack of surface receptors for viral attachment, or postadsorption blocks at different places in the viral growth cycle. Cell transformation by viruses is a field which requires the attention that cell destruction by viruses has received. The transformation of cells in the absence of added virus into cell lines possessing a new viral spectrum of susceptibilities has at times proved to be due to contamination by cells already possessing the new viral susceptibility spectrum.

It is obvious that viruses can act as probes in ferreting out cellular reactions. In essence we have the invasion of one genetic system, the cell, by a foreign genome—the nucleic acid of the virus. The study of the intracellular events which follow upon this invasion has become a fertile field, not only for virologists, but for geneticists as well, and we can confidently predict that both groups will contribute to the elucidation of this basic biological problem.

REFERENCES

1. Evans, C. A.; Byatt, P. H.; Chambers, V. C.; and Smith, W.: Growth of Neurotropic Viruses in Extraneural Tissues; VI. Absence of *in vivo* Multiplication of Poliomyelitis Virus, Types I and II, after Intratesticular Inoculation of Monkeys and Other Laboratory Animals. J. Immun. *72*:348–359, 1954.
2. Kaplan, A. S.: Comparison of Susceptible and Resistant Cells to Infection with Poliomyelitis Virus. Ann. N. York Acad. Sc. *61*:830–838, 1955.
3. Ledinko, N., and Melnick, J. L.: Poliomyelitis Virus in Tissue Culture; III. Susceptibility of Testicular Tissue from Immune Monkeys. Proc. Soc. Exp. Biol. *81*:144–148, 1952.
4. Holland, J. J., and McLaren, L. C.: The Location and Nature of Enterovirus Receptors in Susceptible Cells. J. Exp. M. *114*:161–171, 1961.
5. Holland, J. J.; McLaren, L. C.; and Syverton, J. T.: The Mammalian Cell Virus Retionship; IV. Infection of Naturally Insusceptible Cells With Enterovirus Ribonucleic Acid. J. Exp. M. *110*:65–80, 1959.
6. Mountain, I. M., and Alexander, H. E.: Infectivity of Ribonucleic Acid (RNA) from Type I Poliovirus in Embryonated Egg. Proc. Soc. Exp. Biol. *101*:527–532, 1959.
7. Holland, J. J.: Receptor Affinities as Major Determinants of Enterovirus Tissue Tropisms in Humans. Virology *15*:312–326, 1961.
8. Francis, T., and Chu, L. W.: The Interaction *in vitro* Between Poliomyelitis Virus and Nervous Tissue Constituents. VI. Internat. Cong. Microb., Rome *2*:38, 1953.
9. Joklik, W. K., and Darnell, J. E.: The Adsorption and Early Fate of Purified Poliovirus in HeLa Cells. Virology *13*:439–447, 1961.
10. Kaplan, A. S., and Melnick, J. L.: Multiplication of Virulent Poliovirus in Capuchin Monkey Kidney Cultures Without Microscopically Observed Cytopathogenicity. Proc. Soc. Exp. Biol. *90*:562–565, 1955.
11. Hsiung, G. D., and Melnick, J. L.: Adsorption, Multiplication, and Cytopathogenicity of Enteroviruses (Poliomyelitis, Coxsackie, and ECHO Groups) in Susceptible and Resistant Monkey Kidney Cells. J. Immun. *80*:45–52, 1958.
12. Sabin, A. B.: Genetic, Hormonal and Age Factors in Natural Resistance to Certain Viruses. Ann. N. York Acad. Sc. *54*:936–944, 1952.
13. Vogt, M., and Dulbecco, R.: Properties of a HeLa Cell Culture with Increased Resistance to Poliomyelitis Virus. Virology *5*:425–434, 1958.
14. Darnell, J. E., and Sawyer, T. K.: Variation in Plaque-forming Ability among Parental and Clonal Strains of HeLa Cells. Virology *8*:223–229, 1959.
15. Darnell, J. E., and Sawyer, T. K.: The Basis for Variation in Susceptibility to Poliovirus in HeLa Cells. Virology *11*:665–675, 1960.
16. Ledinko, N.: Blocking of Neutralizing Antibody by Noninfective Poliovirus from Proflavine-treated HeLa Cells. Virology *8*:537–539, 1959.

17. Crowther, D., and Melnick, J. L.: Studies of the Inhibitory Action of Guanidine on Poliovirus Multiplication in Cell Cultures. Virology *15*:65–74, 1961.
18. Ackerman, W. W.; Rabson, A.; and Kurtz, H.: Growth Characteristics of Poliomyelitis Virus in HeLa Cell Culture; Lack of Parallelism in Cellular Injury and Virus Increase. J. Exp. M. *100*:437–450, 1954.
19. Mayor, H. D., and Melnick, J. L.: Intracellular and Extracellular Reactions of Viruses with Vital Dyes. Yale J. Biol. *34*:340–358, 1962.
20. Crowther, D., and Melnick, J. L.: The Incorporation of Neutral Red and Acridine Orange into Developing Poliovirus Particles Making Them Photosensitive. Virology *14*:11–21, 1961.
21. Darnell, J. E., and Eagle, H.: The Biosynthesis of Poliovirus in Cell Cultures. Advances Virus Res. *7*:1–26, 1960.
22. Melnick, J. L.; Hsiung, G. D.; Rappaport, C.; Howes, D.; and Reissig, M.: Factors Influencing the Proliferation of Viruses. Texas Rep. Biol. M. *15*:496–533, 1957.
23. Melnick, J. L.: Characteristics of the Echo Viruses. In Rose, H. M. (Ed.): *Viral Infections of Infancy and Childhood*. New York: Paul B. Hoeber Inc., 1960, pp. 55–77.
24. Hsiung, G. D., and Melnick, J. L.: Morphologic Characteristics of Plaques Produced on Monkey Kidney Monolayer Cultures by Enteric Viruses (Poliomyelitis, Coxsackie, and Echo Groups). J. Immun. *78*:128–136, 1957.
25. Liebhaber, H., and Takemoto, K. K.: Alteration of Plaque Morphology of EMC Virus with Polycations. Virology *14*:502–504, 1961.
26. Wallis, C., and Melnick, J. L.: Unpublished research.
27. Wallis, C., and Melnick, J. L.: Magnesium Chloride Enhancement of Cell Susceptibility to Poliovirus. Virology *16*:122–132, 1962.
28. Vogt, M.; Dulbecco, R.; and Wenner, H. A.: Mutants of Poliomyelitis Virus with Reduced Efficiency of Plating in Acid Medium and Reduced Neuropathogenicity. Virology *4*:141–155, 1957.
29. Lwoff, A.: Factors Influencing the Evolution of Viral Diseases at the Cellular Level and in the Organism. Bact. Rev. *23*:109–124, 1959.
30. McBride, W. D.: Unpublished research.
31. Melnick, J. L., and Benyesh-Melnick, M.: Problems Associated with Live Polio Virus Vaccine and Its Progeny after Multiplication in Man. Pan American Health Organization Scientific Publication No. 50 (Live Poliovirus Vaccines), 1960, pp. 12–30.
32. Wallis, C.; Melnick, J. L.; Ferry, G. D.; and Wimberly, I. L.: An Aluminum Marker for the Differentiation and Separation of Virulent and Attenuated Polioviruses. J. Exp. M. *115*:763–775, 1962.
33. Baluda, M. A., and Goetz, I. E.: Morphological Conversion of Cell Cultures by Avian Myeloblastosis Virus. Virology *15*:185–199, 1961.
34. Kanda, Y., and Melnick, J. L.: In vitro Differentiation of Virulent and Attenuated Polioviruses by Their Growth Characteristics on MS Cells. J. Exp. M. *109*:9–24, 1959.
35. Westwood, J. C. N.; MacPherson, I. A.; and Titmuss, D. H. J.: Transformation of Normal Cells in Tissue Cultures; Its Significance Relative to Malignancy and Virus Vaccine Production. Brit. J. Exp. Path. *38*:138–154, 1957.
36. Sheffield, F. W., and Churcher, G. M.: The Serial Propagation of Poliomyelitis Viruses in Cells Derived from Rabbit Embryo Kidney. Brit. J. Exp. Path. *38*:155–159, 1957.
37. Melnick, J. L., and Habel, K.: Antigenic Relationship of Poliovirus Susceptible Rabbit Kidney Cell Cultures to HeLa and Other Primate Cell Lines. Fed. Proc. *17*:526, 1958.
38. Rothfels, K. H.; Axelrad, A. A.; Siminovitch, L.; McCulloch, E. A.; and Parker, R. C.: The Origin of Altered Cell Lines From Mouse, Monkey, and Man, as Indicated by Chromosome and Transplantation Studies. Canad. Cancer Conf. *3*:189–214, 1959.
39. Brand, K. G., and Syverton, J. T.: Results of Species-Specific Hemagglutination Tests on "Transformed," Nontransformed, and Primary Cell Cultures. J. Nat. Cancer Inst. *28*:147–157, 1962.
40. Clausen, J. J., and Syverton, J. T.: Comparative Chromosomal Study of 31 Cultured Mammalian Cell Lines. J. Nat. Cancer Inst. *28*:117–145, 1962.
41. Barski, G.; Cornefert, F.; and Biedler, J. L.: Caracteres Evolutifs et Permanents de Cellules D'Homme et de Lapin du Cour de Leur Adaptation a la Vie *in vitro*. Ann. Inst. Pasteur Lille *100*:324–336, 1961.
42. Committee on Enteroviruses: Classification of Human Enteroviruses. Virology *16*:501–504, 1962.

Antigens as Genetic Markers

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IMMUNOLOGIC methods have been employed in genetic studies since the early days of immunology. The bacteriologist was one of the first to realize the advantages of these methods and used antigenic markers both to identify bacteria and to follow phenotypic changes. He was able to detect phenotypic changes due to the environment, such as the phase variation of flagellar antigens, and to define the rough-smooth colony variation in antigenic terms.[1] The use of type-specific pneumococcal carbohydrates as antigenic markers in the study of transformation and the utilization of the antigenic structure of the enterobacteriaceae to follow transduction of bacterial characters by bacteriophage[2] are too well known to be discussed.

The introduction of tissue culture techniques for mammalian cells has made it imperative that similar methods be developed either as a means of identification, or as tools for studying environmental influences on the growth of cells. The present paper will discuss the advances that have been made in both fields of endeavor.

IMMUNOLOGIC METHODS IN COMMON USE

The methods used to study antigenic markers of cells grown in tissue culture will depend to a large extent on the nature of the antigens, i.e., whether they are soluble or insoluble, whether on the cell membrane or intracellular, whether strain-specific or cell-specific. The complement fixation test may at times be useful, but since it has few of the advantages of the other tests and most of their disadvantages, this reviewer feels that it will not become a very useful tool for tissue culture work.

Brand and Syverton,[3] for example, found complement fixation unsuitable for their work (see below) since the absorption of antisera made them anticomplementary. This anticomplementary activity, however, can usually be removed by high speed centrifugation as reported by Rapport and Graf.[4] Complement fixation may be helpful in studying the incorporation of viral antigens in previously infected cells that no longer contain demonstrable virus.[5] Moreover, once the methods developed by Rapport and Graf[6] reach the stage where they will be applicable to tissue culture systems, valuable information may be gathered about the distribution of lipid haptens in isolated cell strains. Two such haptens have already been purified and partially characterized.[6,7]

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Whenever the cellular antigens that are involved in the cytotoxic action of an antiserum are to be studied the only suitable method consists of growing the cells in tissue culture and adding the cytotoxic antiserum to these cells while still viable. The injurious effect of the antisera may then be evaluated by different methods.[8–10] Absorption of the antisera with different antigen preparations may ultimately yield information about the chemical nature of the cellular antigens against which the cytotoxic antibodies are directed. The isolation of antigenically transformed cells, i.e., cells of a given culture that are no longer susceptible to the action of a given antiserum, may ultimately be accomplished by the cloning method developed by Puck and his collaborators.[11,12]

The precipitin reaction in aqueous medium can be used if the antigen is soluble and the antiserum is directed against a single cellular antigen—a rare occasion. The precipitin reaction in gel according to Ouchterlony[13] requires large amounts of soluble cell extract, but the antiserum may be directed against more than one antigen since each antigen-antibody system will usually form a separate zone of precipitate in the agar-gel.

The agglutination methods used by the bacteriologist or hematologist have been applied, at times, in a modified form. Since tissue cells are not easily kept in suspension the classical agglutination reaction cannot be used. However, if the cellular membrane antigens are similar to those of the erythrocytes of the same species or individual, erythrocytes can be used for the agglutination reaction, either by themselves[3] (if the antiserum was produced against the tissue cells grown in culture) or in combination with the tissue cells, i.e., the mixed agglutination reaction of Coombs.[14,15]

The use of fluorescent antisera, either against cell membrane antigens, or intracellular antigens, promises to become the most useful tool for the immunologist interested in cells grown in tissue culture. This method[16] enables him to visualize any cellular antigen against which he may have a *specific* antiserum, and to determine whether all cells of a culture produce it. The simultaneous use of multiple antisera (each labeled with a different dye) against different components makes the method even more promising.

RESULTS OBTAINED WITH THESE METHODS

Cytotoxic Antisera—One of the earliest complete descriptions of the effect of cytotoxic antisera on cells grown in tissue culture was presented by Niven[17] in 1929. Since that time many papers have appeared dealing with the use of cytotoxic antisera in the detection of cell-specific antigens or even tumor-specific[18] antigens. Although the most recent findings suggest that most cytotoxic antisera are species-specific,[9,12,19] only a few carefully executed studies have clearly shown that different cells from a given species are affected to a varying degree by some of these antisera.[20–22] The findings of Colter et al.[23] that antisera against nucleoproteins from human leukemia bone-marrow cells were not species-specific, are at variance with the conclusions of most other workers as are their findings that only 3 of the 18 human cell lines tested were affected

by their antisera. Björklund's[24] recent claim that horse antisera against pooled human carcinomas were cancer-specific has not been substantiated.[25]

Gel Diffusion—The double gel diffusion method of Ouchterlony[13] has been used for antigenic analysis when conditions permitted the growth and harvest of large numbers of cells. Many of the cellular antigens are present in small amounts only, and consequently their detection requires soluble extracts having protein concentrations between 10 to 40 mg./ml. Figure 1 shows that human HE p#2 cells still synthesize many human tissue antigens after having been grown for many generations in cortisonized rats, hamsters, and finally in tissue culture. In this instance the antisera were prepared against different human tissues or organs obtained during surgical operating procedures.[26]

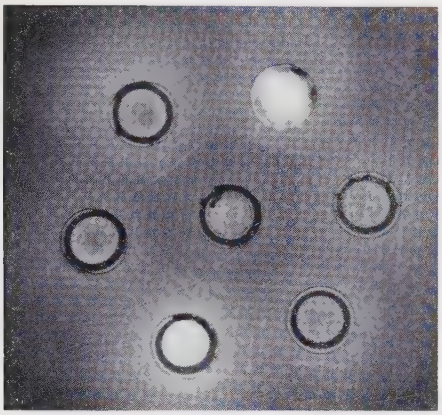

Fig. 1.—Ouchterlony test showing the presence of human antigens in HEp#2 cells grown in tissue culture. Center: HEp#2 cell extract. Peripheral wells: antihuman tissue sera. Note that HEp#2 does not react with all antisera.

However, it is possible to produce precipitating antisera against cells grown in culture, and such antisera may then be used to follow the antigenic composition of future cells from the same cell line or to compare these cells with other cells grown in tissue culture. It is important to absorb these antisera with the serum that was used in the medium. Elsewhere I have presented examples of the reactivity of precipitating antisera against cultured cells.[27] The double gel diffusion technique, moreover, has the advantage that structural changes of a cellular antigen may be detected. For example, if such an antigen were to lose some of its original antigenic determinants this change would appear as a spur between the lines produced by the original antigen and the changed antigen.

Agglutination Reaction—This method was developed by Brand and Syverton[3] to determine the species of origin of cultivated mammalian cells. It depends on several assumptions, most of which have been shown to be correct. The first assumption is that all tissue cells of a given organism share species-specific antigens with the erythrocytes of that organism. The second assump-

tion is that most animals used for immunization will respond to the inoculation of tissue culture cells with the production of species-specific agglutinins. The third assumption is that the erythrocytes from different animals of the same species will be agglutinated to the same degree.

The method consists of inoculating rabbits, guinea pigs, or other animals with the cultured cells. Pre-immunization sera should be obtained. After a series of injections and trial bleedings the animals are exsanguinated and the antisera tested for specificity. Since most pre-immunization sera will agglutinate erythrocytes from many mammalian species, it is important to compare the antiserum with the pre-immunization serum. An increase in titer only against the erythrocytes of the species from which the cultured cells were derived is essential. This comparison is important in another respect, as Brand and Syverton[3] found that erythrocytes from different animals of the same species were agglutinated to different degrees. Using antisera against a large number of cultured mammalian cell lines, it was shown that these antisera agglutinated only erythrocytes of the species from which the cells had been derived[3,28] (difference in titer of pre-immunization bleeding and immune serum). The inoculation of so-called transformed cells did not result in antisera capable of agglutinating erythrocytes of the species from which the cultured cells were believed to have been derived.[28] Since the species-specific antigens were found to be very stable and present in cultured cells after many passages under different environmental conditions, including the presence of carcinogens, Brand and Syverton[28] concluded that the "transformed" cells were in reality contaminating or mislabeled cells of different origin from the one presumed. The fact that the "transformed" cells turned out to be either mouse cells or human cells (i.e., the cells most commonly carried in tissue culture) supported this conclusion, as did chromosome analysis of the cells. The agglutination method is simple and is probably reliable as long as it is realized that some of the assumptions listed above may not hold in all instances. (Stulberg et al.,[29] for example, did not obtain good hemagglutinating sera in guinea pigs after inoculation with mouse or hamster cells.) The following points should be considered in preparing anticell sera. According to Brand and Syverton,[3] it is important to limit the number of inoculations to a minimum since repeated booster inoculations may result in decreased specific hemagglutination titers. The use of guinea pigs larger than 250 gm. seems to result in antisera with more cross reactivity.

Mixed Agglutination Reaction—The agglutination reaction described above uses erythrocytes as the test system and the tissue culture cells as immunizing antigens. R. R. A. Coombs[14] developed a test system in which both the tissue culture cells and the erythrocytes are used in the reaction. The test again depends on the presence of common antigens on the membranes of the erythrocytes and the tissue cells. In one of their first attempts to demonstrate the applicability of this test, Coombs and his co-workers[15] used epidermal cells obtained from human skin. These cells were obtained from individuals belonging either to blood group A or B. When the tissue cells belonging to an

individual of group B were treated with an anti-B serum, the antibodies combined with B receptors on the cell membrane. Since these antibodies have two combining sites, one site remained free. The addition of group B erythrocytes resulted in a mixed agglutination which was easily visualized under the microscope. Using antisera against the many RBC antigens, cells grown in tissue culture can be typed. For example, HeLa cells contain H, M, N, and T_{ja}, but lack the Rh, A and B isoantigens.[30]

The mixed agglutination reaction was also used to determine the presence of Forssman antigen[31] in cells grown in tissue culture.[32] Cells from animal species that were Forssman-positive invariably contained the heterophile antigen, even when grown for many days in media lacking Forssman antigens. Negative cells, however, would adsorb the antigen to their membranes. Mouse L cells, isolated by Earle more than 20 years ago, no longer contained the Forssman antigen.[32] Unfortunately, we do not know when the antigen was lost, or even whether the original cells or the mouse from which they were isolated contained this antigen.

The mixed agglutination reaction, like the agglutination reaction, can be used to determine the species of origin of cells grown in culture.[33] Coombs and his co-workers used antisera—usually prepared in rabbits—against erythrocytes of different species. Cross reactions, though weaker than the homologous reaction, complicated this work, especially since absorption was frequently found to be ineffective.

Figures 2a, 2b, 3a, and 3b, for which I am grateful to Dr. Coombs, illustrate such a recognition test. Figure 2a shows a positive test for pig kidney cells using anti-pig erythrocyte serum, and Figure 2b is the negative control. Figure 3a represents a similar test for human cells (KB cells) with a specific antihuman red blood cell serum; Figure 3b is the negative control.

The advantage of the mixed agglutination technique resides in its ability to type tissue cells with the many specific anti-RBC sera now available for human, mouse, rabbit, and bovine erythrocytes. It is also possible to observe antigenic changes which may occur under different conditions. Even antisera that do not agglutinate can be used since it is possible to use mixed antiglobulin reactions.[14] The disadvantage of the method is its dependence on membrane antigens.

Immunohistochemical Methods—These methods have become available as a result of Coons'[34] work and overcome the above disadvantage. In this method, antisera are prepared against the desired cellular antigens; these can be either membrane, nuclear, or cytoplasmic. These antisera are then labeled with a fluorescent dye, and the cells are stained with this labeled reagent. The technical procedures have been described in detail elsewhere.[16]

Until recently these techniques have been limited to tissues taken directly from the animal and they were used to define, among others, the location of blood group antigens[35] and Forssman antigens.[36] Similarly, it has been possible to detect cells responsible for the synthesis of γ_2-globulin,[37] macroglobulin,[38] rheumatoid factor,[38] CRP_x,[39] and thyroglobulin.[40] Hiramoto et al.[41,42]

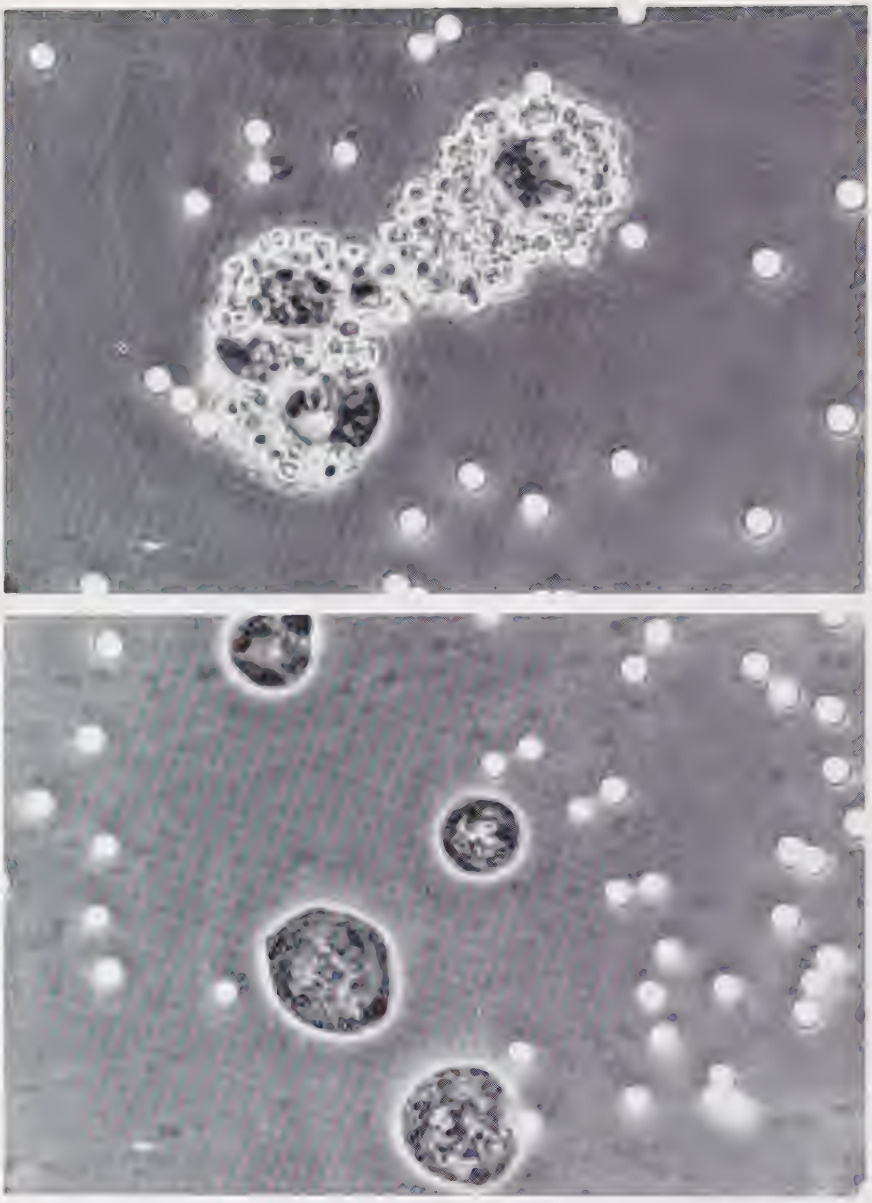

Fig. 2.—Mixed agglutination. (a) Pig kidney cells and pig erythrocytes. Positive reaction with specific anti-pig RBC serum. (b) Negative control.

were among the first to apply this new immunohistochemical method to the identification of cells grown in tissue culture. Their interest centered on the ability of cultured muscle cells to produce myosin. Specific antisera were prepared against myosin or connective tissue antigens of either mouse or human origin. When such antisera were labeled with fluorescent dyes, it was possible

to demonstrate myosin in muscle cells among fibroblasts producing connective tissue antigens in outgrowths of skeletal muscle explants. The presence of both antigens was demonstrated by staining the same preparation with a paired label, i.e., one antiserum was labeled with fluorescein isothiocyanate (green) and the other with tetramethyl rhodamine isothiocyanate (red).[41]

At first, during the early stages of cultivation, the muscle cells stained only

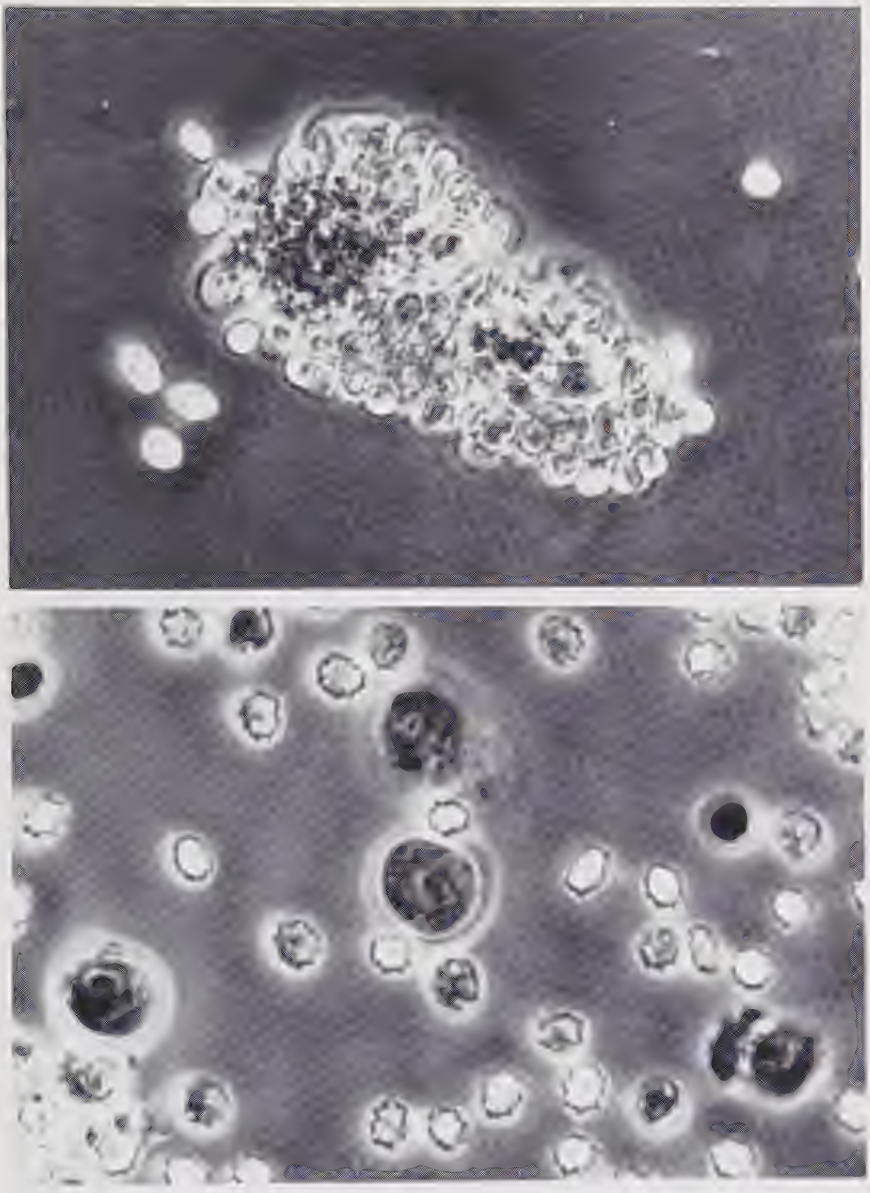

Fig. 3.—Mixed agglutination. (a) Human KB cells and human erythrocytes. Positive reaction with antihuman RBC serum. (b) Negative control.

with the antimyosin serum, but on continued growth in tissue culture, connective tissue antigen was also produced by these cells. Samples of embryologic rhabdomyosarcoma obtained at necropsy reacted with the antimyosin serum, but the ability of the cells to form myosin was lost upon cultivation.

The method could also be used to differentiate mouse cells from human cells, even when the former comprised only a small percentage of the total sample.[42] Figure 4 illustrates such an experiment.

Fig. 4.—Human tissue culture cells (cancer of gallbladder) purposely contaminated with a few L929 cells of mouse origin. The section was treated with fluorescein-labeled, anti-mouse connective tissue reactive antibody (absorbed with human liver) and subsequently with tetramethylrhodamine-labeled, antihuman connective tissue reactive antibody (absorbed with mouse liver). The paired labeled procedure showed two mouse cells which fluoresced brightly green while all background human cells fluoresced orange. (From Hiramoto et al.[42])

Stulberg et al.[29] have used immunofluorescence to determine the species of cells grown in tissue culture, using antimembrane antisera produced as described by Brand and Syverton.[3]

The results described above indicate that the use of immunologic methods for the study of mammalian cells grown in tissue culture may become valuable tools in the future. An extension of their use will depend primarily on two conditions: In the first place, it will be necessary to develop enough suitable antisera against many more cellular antigens than are now available. To avoid a monumental confusion brought about by different laboratories using antisera with different properties, a central clearing house will have to be established where samples of antisera can be kept for future comparison with similar reagents from other laboratories. Secondly, media and methods will have to be developed which will permit the culture of cells without loss of morphologic or functional properties, thus making available a much larger variety of cells.

If these conditions can be met in the near future, many important problems dealing with mammalian cells may be attacked and solved. Some of these problems will be: (1) the effect of environmental factors on the antigenic composition of the cell; (2) transformation of cells by DNA and its effect on cellular antigens; (3) the production of different γ-globulins by clones of plasma cells obtained from the same animal; (4) changes in cellular antigens mediated by viruses; and (5) the effect of carcinogens on the antigenic structure of the cells.

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REFERENCES

1. Dubos, R. J.: *The Bacterial Cell.* Cambridge: Harvard University Press, 1947, pp. 1–460.
2. Lederberg, J.: Viruses, Genes, and Cells. Bact. Rev. *21*:133–139, 1957.
3. Brand, K. G., and Syverton, J. T.: Immunology of Cultivated Mammalian Cells; I. Species Specificity Determined by Hemagglutination. J. Nat. Cancer Inst. *24*:1007–1019, 1960.
4. Rapport, M. M., and Graf, L.: Immunochemical Analysis Based on Complement Fixation. Ann. N. York Acad. Sc. *69*:608–632, 1957.
5. Vogt, M., and Dulbecco, R.: Studies on Cells Rendered Neoplastic by Polyoma Virus; The Problem of the Presence of Virus-Related Materials. Virology *16*:41–51, 1962.
6. Rapport, M. M.; Graf, L.; Skipski, V. P.; and Alonzo, N. F.: Immunochemical Studies of Organ and Tumor Lipids. Cancer *12*:438–445, 1959.
7. Graf, L.; Rapport, M. M.; and Brandt, R.: Immunochemical Studies of Organ and Tumor Lipides; X. The Presence of a Novel Lipide Hapten, Cytolipin G, in Tumors and Tissues of the Gastrointestinal Tract. Cancer Res. *21*:1532–1536, 1961.
8. Salk, J. E., and Ward, E. N.: Some Characteristics of a Continuously Propagating Cell Derived from Monkey Heart Tissue. Science *126*:1338–1339, 1957.
9. Miller, D. G., and Hsu, T. C.: The Action of Cytotoxic Antisera on the HeLa Strain of Human Carcinoma. Cancer Res. *16*:306–312, 1956.
10. Reif, A. E., and Norris, H. J.: A System for Quantitative Determination of Cytotoxic Activity of Antisera to Ascites Tumor Cells. Cancer Res. *20*:1235–1244, 1960.
11. Puck, T. T.; Marcus, P. I.; and Cieciura, S. J.: Clonal Growth of Mammalian Cells *in vitro*. J. Exp. M. *103*:273, 1956.
12. Oda, M., and Puck, T. T.: The Interaction of Mammalian Cells with Antibodies. J. Exp. M. *113*:599–610, 1961.
13. Ouchterlony, O.: Diffusion-in-Gel Methods for Immunological Analysis. Progr. Allergy *5*:1–78, 1958.
14. Coombs, R. R. A.: The Mixed Agglutination Reactions in the Study of Normal and Malignant Cells. Cancer Res. *21*:1198–1202, 1961.
15. Coombs, R. R. A.; Bedford, D.; and Rouillard, L. M.: A and B Blood-Group Antigens on Human Epidermal Cells Demonstrated by Mixed Agglutination. Lancet *1*:461–463, 1956.
16. Mellors, R. C.: Fluorescent-Antibody Method. In Mellors, R. C. (Ed.): *Analytical Cytology*. New York: McGraw-Hill Book Company, Inc., 1959, pp. 1–67.
17. Niven, J. S. F.: The Action of Cytotoxic Antiserum on Tissue Cultures. J. Path. Bact. *32*:527–550, 1929.
18. Lumsden, T., and Kohn-Spyer, A. C.: Tumor Immunity. J. Path. Bact. *32*:185–194, 1929.
19. Goldstein, M. N.: The Cross Reactions of Antisera Prepared Against Four Human Cell Strains in Continuous Tissue Culture. J. Immun. *79*:113–117, 1957.

20. Harris, M.: Specificity and Mode of Action of Cytotoxins Produced Against Alien Transplants in Rats. J. Exp. Zool. *107*:439–453, 1948.
21. Bassett, C. A. L.; Campbell, D. H.; Evans, V. J.; and Earle, W. R.: The Cytotoxic Activity of Rabbit Immune Globulin Prepared from Tissue Cultures of Human Skin and Whole Human Placenta. J. Immun. *78*:79–93, 1957.
22. Ehrlich, G., and Halbert, S. P.: The Effects of Anti-Cornea and Anti-Heart Serum on Cultured Cells of Rabbit Cornea and Other Tissues. J. Immun. *86*:267–281, 1961.
23. Colter, J. S.; Defendi, V.; Wallace, R. E.; and Bird, H. H.: Immunological Studies with Nucleoproteins from Tissue-Culture Cells; I. Nucleoproteins from Human Leukemic Bone-Marrow (MCN) Cells. J. Nat. Cancer Inst. *20*:1141–1155, 1958.
24. Björklund, B., and Björklund, V.: Antigenicity of Pooled Human Malignant and Normal Tissues by Cyto-Immunological Technique; Presence of an Insoluble, Heat-Labile Tumor Antigen. Internat. Arch. Allegy, Basel *10*:153–184, 1957.
25. Goldstein, M. N., and Hiramoto, R.: Cytotoxicity of a Horse Antihuman Cancer Serum for Normal and Malignant Cells *in vitro*. J. Nat. Cancer Inst. *27*:487–501, 1961.
26. Korngold, L.: The Distribution and Immunochemical Properties of Human Tissue and Tumor Antigens. Ann. N. York Acad. Sc. *69*:681–697, 1957.
27. Korngold, L.: Tissue-Specific Antigens. National Cancer Institute Monograph 2, 1960, pp. 57–71.
28. Brand, K. G., and Syverton, J. T.: Results of Species-Specific Hemagglutination Tests on "Transformed," Nontransformed, and Primary Cell Cultures. J. Nat. Cancer Inst. *28*:147–157, 1962.
29. Stulberg, C. S.; Simpson, W. F.; and Berman, L.: Species-Related Antigens of Mammalian Cell Strains as Determined by Immunofluorescence. Proc. Soc. Exp. Biol. *108*:434–439, 1961.
30. Kelus, A.; Gurner, B. W.; and Coombs, R. R. A.: Blood Group Antigens on HeLa Cells Shown by Mixed Agglutination. Immunology *2*:262–267, 1959.
31. Hawes, M. D., and Coombs, R. R. A.: Cellular Distribution of Forssman (Membrane) Antigen in the Guinea Pig Determined by the Mixed Agglutination Reaction. J. Immun. *84*:586–589, 1960.
32. Coombs, R. R. A.; Daniel, M. R.; Gurner, B. W.; and Kelus, A.: Recognition of the Species of Origin of Cells in Culture by Mixed Agglutination; II. Use of Heterophile (Anti-Forssman) Sera. Internat. Arch. Allergy, Basel *19*:210–226, 1961.
33. Coombs, R. R. A.; Daniel, M. R.; Gurner, B. W.; and Kelus, A.: Recognition of the Species of Origin of Cells in Culture by Mixed Agglutination; I. Use of Antisera to Red Cells. Immunology *4*:55–66, 1961.
34. Coons, A. H., and Kaplan, M. H.: Localization of Antigen in Tissue Cells; II. Improvements in a Method for the Detection of Antigen by Means of Fluorescent Antibody. J. Exp. M. *91*:1–13, 1950.
35. Glynn, L. E.; Holborow, E. J.; and Johnson, G. D.: The Distribution of Blood-Group Substances in Human Gastric and Duodenal Mucosa. Lancet *2*:1083–1088, 1957.
36. Tanaka, N., and Leduc, E. H.: A Study of the Cellular Distribution of Forssman Antigen in Various Species. J. Immun. *77*:198–212, 1956.
37. Ortega, L. G., and Mellors, R. C.: Cellular Sites of Formation of Gamma Globulin. J. Exp. M. *106*:627–640, 1957.
38. Mellors, R. C.; Nowoslawski, A.; Korngold, L.; and Sengson, B. L.: Rheumatoid Factor and the Pathogenesis of Rheumatoid Arthritis. J. Exp. M. *113*:475–484, 1961.
39. Kushner, I., and Kaplan, M. H.: Studies of Acute Phase Protein; I. An Immunohistochemical Method for the Localization of Cx-reactive Protein in Rabbits; Association with Necrosis in Local Inflammatory Lesions. J. Exp. M. *114*:961–974, 1961.
40. Beutner, E. H., and Witebsky, E.: Studies on Organ Specificity; XIV. Immunofluorescent Studies of Thyroid Reactive Antibodies in Human Sera. J. Immun. *88*:462–475, 1962.
41. Hiramoto, R.; Cairns, J.; and Pressman, D.: Immunohistochemical Studies on the Antigenic Relationship of Some Muscle Tumors to Normal Skeletal Muscle Cells. J. Nat. Cancer Inst. *27*:937–947, 1961.
42. Hiramoto, R.; Jurandowski, J.; Bernecky, J.; and Pressman, D.: Immunohistochemical Identification of Tissue Culture Cells. Proc. Soc. Exp. Biol. *108*:347–353, 1961.

Mating of Somatic Cells *In Vitro*

Boris Ephrussi, D.Sc., and Serge Sorieul, M.S.

THERE are several reasons why the establishment of methods for the genetic analysis of somatic cells of higher organisms is of great interest. In our opinion, among the more general ones are the following:

1. As pointed out by Pontecorvo,[1] genetic analysis of somatic cells could, theoretically, replace experimental breeding and, if combined with tissue culture methods, would tremendously accelerate the genetic study of species where experimental breeding is slow or, like in man, impossible for other reasons.

2. Genetic analysis of somatic cells is obviously necessary in the study of embryonic differentiation, i.e. of the process by which the various cell lines derived from a single cell, the fertilized ovum, become different even though they apparently contain the same, initially complete genetic endowment. Both environmental and genetic factors are obviously involved in this fundamental and mysterious process which, to be understood, must be studied on the very cells which are the seat of differentiation.

3. The availability of techniques for the genetic analysis of somatic cells could also be of great assistance in the study of the neoplastic process, in particular of virus-induced neoplasia and, more generally, in the study of cell-virus relationship.

All this granted, what are the requirements of the genetic analysis of somatic cells, and why is it that the necessary tools, which are just beginning to be forged, have not been developed earlier?

As Professor Ryan emphasized in his opening lecture, "The most important methods to be developed for the exploitation of mammalian cell genetics are those which will allow study of the recombination of genes."[2] Now, as long as the study of gene recombination depended entirely on the analysis of the products of sexual reproduction, there seemed to be no hope for a direct study of genetics of somatic cells. New hope in this connection was awakened by the developments in microbial genetics, of which one of "the main contributions is the realization that sexual reproduction is not the only process for bringing together and recombining genetic structures from different lines of descent. A corollary is that we are no longer limited to the use of sexual reproduction as the only tool for genetic analysis." (Pontecorvo[1])

What Pontecorvo refers to in this quotation are, on the one hand, the processes of transformation, transduction, and lysogeny, well known in bacteria,

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which assure the transfer of parts of the genetic material of one cell to another one and, on the other hand, the much less known parasexual cycle which he described in filamentous fungi and the essence of which is, as its name implies, to achieve by other means a result equivalent to that of sexual reproduction, notably, the recombination and segregation of genes.

As Pontecorvo[1] states, "the parasexual cycle in filamentous fungi, typically in *Aspergillus* and *Penicillium,* consists of the following elements: (1) Formation of diploid nuclei by rare, and probably accidental, nuclear fusion in a multinucleate mycelium in which the nuclei are usually haploid. . . . (2) Multiplication of the diploid nuclei side by side with the haploid ones. . . . (3) Mitotic segregation, either by processes which leave the diploid condition unaffected, or by processes which lead to haploidization of the diploid nuclei."

We might add more specifically that, as shown by Pontecorvo and coworkers,[3] mitotic segregation in vegetative cells may be the result of either mitotic crossing over or of the related phenomena of nondisjunction and haploidization by successive, accidental loss of chromosomes.

Guided by the conviction that at least the two latter mechanisms are bound to occur in somatic cells of mammals, Pontecorvo, a few years ago, switched his activity from *Aspergillus* to man and, using tissue culture techniques, is trying to map human chromosomes via mitotic segregation. While it is too early to say whether his optimism was justified, it is certainly not unshared. In fact it is exceeded by that of Lederberg,[4] as illustrated by the following quotation from his summing-up talk at the 1958 Gatlinburg Symposium on "Genetic Approaches to Somatic Cell Variation":

> I am still rather puzzled why biologists show such a strong antisexual bias in the consideration of somatic cells. On the other hand, I think I would have been the first to ridicule the fantasy that viruses might carry bits of genetic material from one cell to another in a transductive process, and yet suggestions of this kind seem to be accepted with great gullibility. Projections for future experimentation on somatic cells have invoked transductive phenomena almost to the exclusion of mating. After all, if we combine Stern's discussion with Hauschka's, we will see that every single one of the unit processes needed for the technical handling of mating has been documented in somatic cells. True, they have not been serially documented on a given set of cells under experimental control. But we have reports of the fusion of somatic cells. We know that nuclei of binucleate cells can fuse, if only by coalescence of the spindles at the next mitosis. We know we can have somatic segregation as well as mitotic crossing over. Fifteen years ago we had a much more negative outlook with regard to the possibility of Mendelian analysis with such organisms as bacteria, viruses, and *Penicillium* than we now have for somatic cells.

It can be seen that Lederberg not only believed that somatic segregation can be made use of in the genetic analysis of somatic cells but also believed in the possibility of mating somatic cells, an equivalent of phase I of the typical parasexual cycle.

Our paper is concerned mainly with the confirmation of this view which, we believe, increases the hopes of a rapid development of somatic cell genetics. Since our findings have been reported thus far only in two very brief notes[5,6] and since they have been received in some quarters with a mixture of skepticism and enthusiasm, we shall describe them here in detail.

The starting point of our own work on mammaliam cell hybridization was a brief report by Barski and co-workers[7] published in October 1960. Having observed that two lines of mouse fibroblasts (derived from Sanford's so-called "high" and "low" cancer lines) differ in several morphological characteristics, including the histological type of the tumor produced by implantation of the cells, as well as in karyotype, Barski, in search of evidence for the occurrence of "transfer of characters," set up mixed cultures of the two lines. One of us (S.S.), at that time in Dr. Barski's laboratory and in charge of the karyological identification of the cells of the two lines, observed the occurrence, in the mixed cultures, of a new cell type exhibiting the karyological characteristics of a hybrid, namely the presence of marker chromosomes of the two lines and a total number of chromosomes expected in the fusion products of pairs of cells from the two lines.

Although the karyotype of these cells appeared to us to provide conclusive proof of the occurrence of hybridization, presumably by cell fusion as the initial step, we set out in January 1961 to repeat Barski's experiments and, if possible, to confirm his observations under better defined conditions.

In this first series of experiment, we used the "high" and "low" cancer lines of Sanford and co-workers,[8] known respectively as lines NCTC 2472 and NCTC 2555; these were kindly supplied to us by Drs. K. K. Sanford, W. R. Earle, and G. Barski. For brevity, we shall refer to them hereafter as N-1 and N-2. It should be pointed out here that our experiments were performed with the original lines of Sanford, while in Barski's experiments either one or both of the lines used were derived from tumors produced by the inoculation of the original strains into C3H mice. The origin of the two lines, N-1 and N-2, will be recalled later in this paper as it will be more instructive then.

The karyotypes of the two lines, both hypotetraploid, are very different and proved to be very stable, both in cultures of each line separately and in the mixed cultures. These karyotypes, as determined on cells in the mixed cultures, are given in Table 1.

TABLE 1.—*Karyotypes of Lines NCTC 2472 (N-1) and NCTC 2555 (N-2)*

Karyotypic Characteristics	N–1	N–2
Total number of chromosomes, modal	55	57
Total number of chromosomes, variation extremes	51–59	51–64
Number of biarmed chromosomes, modal	1	15
Number of biarmed chromosomes, variation extremes	1–3	11–16
Number of extra long telocentrics, modal	1.5	0
Number of extra long telocentrics, variation extremes	1–3	0

This Table gives the values for *s* cells only. Both cell lines contain in addition some 2*s*, 4*s*, etc. cells and, in the N-1 line in particular, the proportion of these cells is rather high. However, it can be seen from Table 1 that the cells of the two lines can be unmistakably identified in a mixed culture by the presence in N-1 cells of at least one and mostly two extra long chromosomes (one

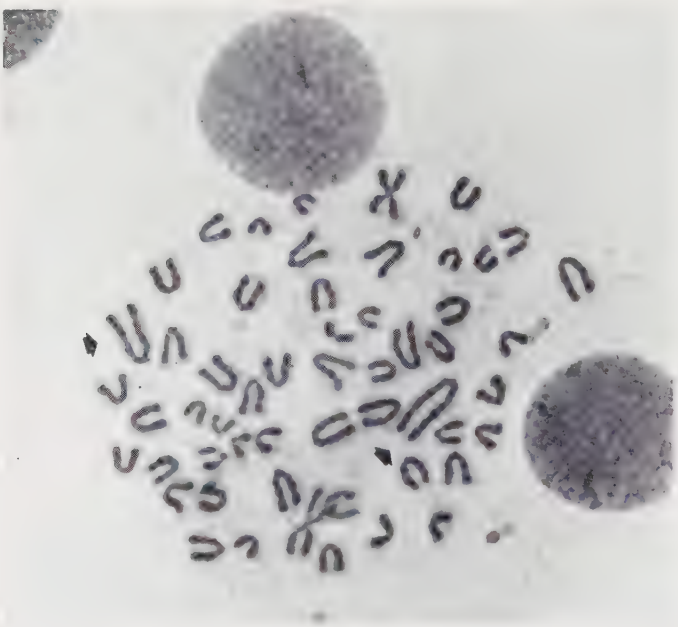

Fig. 1.—Cell of line NCTC 2472 (N-1). Arrows indicate the two extra long chromosomes.

Fig. 2.—Cell of line NCTC 2555 (N-2). Notice the numerous biarmed chromosomes. Arrow indicates a characteristic short metacentric marker.

of which presents a characteristic submedian secondary constriction) and by the presence in N-2 cells of a high number of metacentrics, some of which represent good markers. These features will be recognized in the microphotographs, Figures 1 and 2.

Mixed cultures were initiated with 2×10^5 cells of each type. The cells were grown on glass in 60 ml. pharmaceutical bottles containing 6 ml. of medium. Two different media were used, NCTC 109 and a modification of medium 199 (commercially produced by the Pasteur Institute in Paris and reinforced with a vitamin mixture). To both media 10 percent of heat-inactivated horse serum was added. One of the mixtures in each medium was transferred after trypsinization, the other after mechanical dispersion of the cells. The cultures were transferred once a week and two renewals of medium were performed between transfers. All cultures were incubated at 37° C. in an atmosphere of 5 percent CO_2 in air.

In three of the four mixtures, cells of strain N-1 proved to have a distinct selective advantage over N-2 cells. Nevertheless, hybrid cells appeared about two and one-half months after the beginning of the experiment in all four series, and their proportion rapidly increased in three of the four mixed cultures. Clearly, in these cases the hybrids had a distinct selective advantage over the N-1 cells. The hybrid cells were recognized by the total number of chromosomes and the number of metacentric chromosomes expected from the fusion of the hypotetraploid *s* cells of the two strains, as well as by the presence of marker chromosomes. In the hybrid cells appearing in the mixture

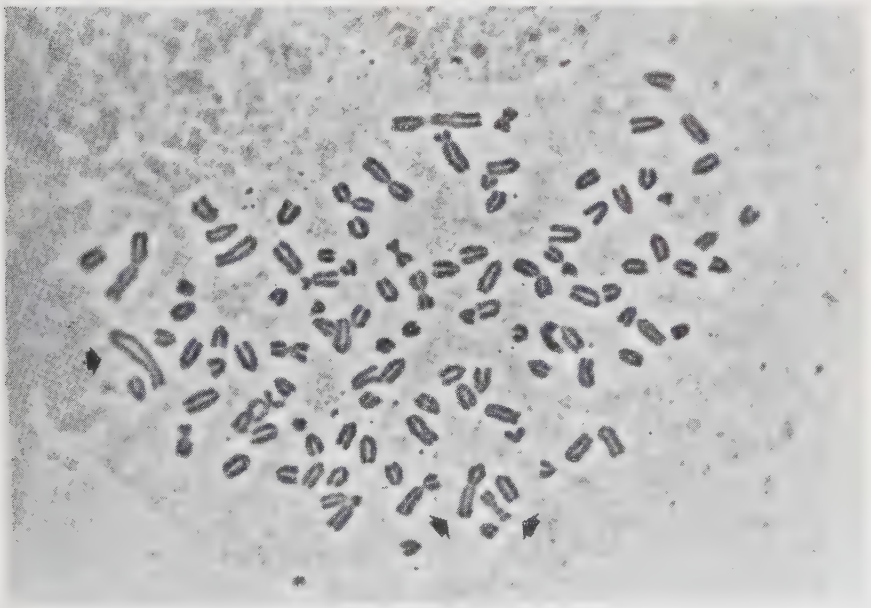

Fig. 3.—Hybrid cell H-109t. Arrows indicate the two extra long telocentric chromosomes, markers of line N-1, and the short metacentric marker of line N-2.

grown in medium 109 and trypsinized for transfer (hybrid H 109t), the modal number of chromosomes, based on the study of 50 mitoses, was 114 (variation extremes: 104–120); the modal number of metacentrics was 17 (variation extremes: 12–19); and the modal number of extra long chromosomes was 2 (variation extremes 1–3). Figure 3 represents a cell of hybrid H 109t. This cell carries 110 chromosomes, of which 16 are metacentrics. Among the latter, the characteristic small metacentric marker of N-2, and the extra long chromosomes of line N-1, one of them carrying the constriction, can be clearly recognized in the figure.

Figure 4 describes the evolution of the population H 109t of which the hybrid cell just shown is an example.* It illustrates what we said before, namely that in the culture, initiated with equal numbers of the two cell types, the

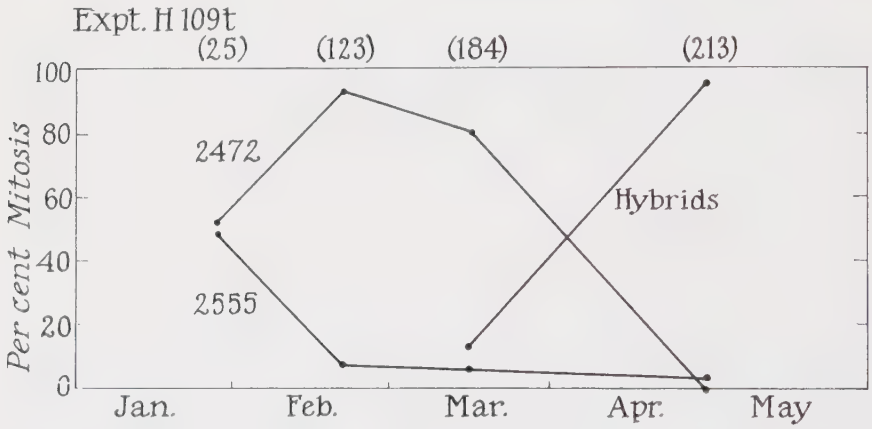

Fig. 4.—Evolution of the mixed population H-109t. Number of analyzed mitoses in parentheses.

N-2 cells rapidly went down; that, therefore, at the beginning N-1 had a distinct selective advantage; and that the hybrid cells, which were detected about March 15, started rapidly increasing, thereby eliminating N-1. Eventually this culture became a pure culture of hybrid cells. It was before this stage, however, that we published our first short note[5] and because of the skepticism that it produced, we are now giving the full description of the course of events. The skeptics suspected that in our microscopic preparations, prepared according to the technique of T. C. Hsu, the appearance of hybrids was an artifact produced by the superposition of two cells of the two types. In this connection it is interesting to notice that no hybrid cells were detected at the time when the two cell types were present in the population in equal proportions and when, therefore, the chances of the hypothetical artifact were at a maximum. This in fact is the first argument for the reality of the hybrids, and later it

* All percentages are deduced from the proportion of metaphases characteristic of the two parental lines; this is obviously an imperfect procedure but the best one available.

was supplemented by a much more powerful one, namely our ability to isolate pure clones of hybrid cells by the Puck cloning procedure. We have thus isolated several hybrid clones and, incidentally, it is interesting to notice that in cloning by the Puck procedure, the hybrid clones are rapidly recognizable by their bigger size (resulting from bigger size and more rapid growth of the cells), a fact which is consistent with what was said earlier about the selective advantage of the hybrids over the N-1 and N-2 cells.

These first results naturally led us to wonder whether our observations could be extended to other cell lines, and whether hybridization could be achieved between mouse cells whatever their relationship, i.e. whatever their genotype. These questions were raised in particular because of the close relationship between lines N-1 and N-2 which represent *two different clones ultimately derived from a single cell of subcutaneous connective tissue of a C3H mouse.*

We therefore set up five new mixtures involving the following three lines:* (1) NCTC 2445, another clone derived from the cell which gave rise to lines N-1 and N-2; (2) LM, a clone of L cells derived from another C_3H mouse; and (3) Py 198-1, a permanant clone derived from a Swiss mouse and converted *in vitro* to the neoplastic condition by polyoma virus by Dr. Renato Dulbecco. The first two lines were grown in mixed cultures with N-1 and N-2, the third with N-2 only.

Of course, another criterion was also used in the selection of these lines: only lines were selected whose karyotypes would enable us to recognize the hybrid cells if such were produced.

Mixed cultures of initially identical numbers of cells of each pair were inoculated in different media (containing 10 percent of serum) selected to prevent too rapid an overgrowth of one of the components of the mixed population by the other. Otherwise, the culture technique was the same as in our first experiment.

Of the listed cultures, only one resulted in success, namely N-2 + Py 198-1 in medium 109 + 10 percent calf serum (culture M-109). The karyotype of N-2 has already been described. That of Py 198-1 (also established in the mixed culture) is nearly tetraploid. The modal number of chromosomes, all acrocentric, is 74, with variation extremes 72–76. Some of the chromosomes, usually two or three, are characterized, like some normal mouse chromosomes, by the presence of proximal heterochromatic differentiations (Figure 5), never observed in N-2 cells.

On the simplest assumptions, the fusion of cells of the two lines, N-2 and Py 198-1, should result in a hybrid karyotype characterized by the following numbers: 131 chromosomes of which 15 metacentric, and 2–3 of the just described "markers" of Py 198-1. Actually, the study of the first 24 hybrid metaphases detected showed the following numbers: 130 (109–134) chromo-

* These lines were kindly supplied to us by Drs. K. K. Sanford, T. C. Hsu, and R. Dulbecco, respectively.

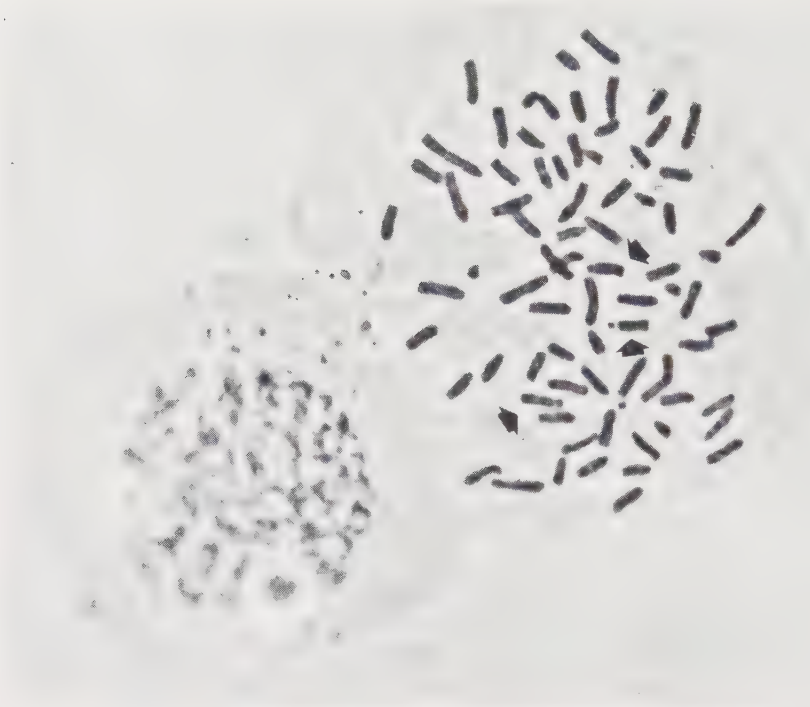

Fig. 5.—Cell of line Py 198-1. Arrows indicate the acrocentric chromosomes used as markers.

somes, 14 (10–17) metacentrics, 2 acrocentric markers of Py 198-1, in very good agreement with expectation.

Figure 6 is a microphotograph of the karyotype of one, rather low, cell of this (M-109) hybrid population.

In this case, as in the case of the mixed N-1 + N-2 populations, the proportion of hybrid cells rapidly increased after they were first detected at the 1 p. 1,000 level, four and one-half months after the beginning of the experiment, reaching 10 percent about four weeks later, and practically 100 percent one and one-half months thereafter. Clearly, here too, the hybrid cells had a distinct selective advantage over the cells of the "parental" lines, and this was again confirmed by the easy isolation of several clones of hybrid cells.

The significant conclusion to be drawn from our second successful cross is that identity of origin is not a prerequisite for mating. As pointed out earlier, lines N-2 and Py 198-1 are derived respectively from C_3H and Swiss mice, i.e., from animals not only of nonidentical, but certainly of very different genotypes.

The failure of the other attempted crosses should not be taken to mean that mating between cells of these pairs of lines does not take place. In the first place, we are dealing with a negative result. Moreover, in some of the cases, one of the parental lines was rapidly overgrown by the other in spite of our efforts to maintain them in equilibrium. Lastly, it is not certain *a priori*

that all hybrids would have a selective advantage over the parental lines; this is the only situation in which we are thus far able to detect the hybrids.

Be this as it may, one cannot help wondering about the significance of the fact that both of our successful crosses involve a common parent, namely line N-2. Is this purely accidental? We are hopeful that the answer to this question will be forthcoming soon because it may enlighten us on the prerequisites of the "mating" process. In this connection the following remarks may be of some interest.

1. There is a peculiar point in the history of line N-2 which may be worth mentioning. As recalled above, this line is ultimately derived from the same cell as line N-1, and several other "high" and "low" cancer-inducing sublines. Moreover, N-2 is a subclone derived by single-cell isolation from one of the "low" cancer lines. Now, the singular feature of line N-2 is that, according to Sanford et al.,[9] the cell which gave rise to this subclone was binucleate at the moment of its isolation, but that this peculiarity disappeared before the cell divided; presumably the two nuclei had fused. This observation raises the question whether, and in fact suggests the possibility that, tendency to fusion of nuclei may be a genetic property of this particular clone, N-2, which would then be, so to say, a rather unique "mater" line, just as K-12 was for a long

Fig. 6.—Hybrid cell M-109. The small arrows indicate acrocentric chromosomes of line Py 198-1; the large one indicates the small metacentric marker of line N-2.

time a unique "mater" line of *E. coli*. This possibility, which is a pessimistic one because it would make mating a much more restricted genetic tool, is not an incredible one if one thinks in particular of the mechanisms assuring diploidization by nuclear fusion established in some cases in nature, and which are almost certainly genetically controlled, for example, in species which reproduce by facultative parthenogenesis.

2. This, however, is not the only possibility to be considered, for there is another feature, common to our two successful crosses, aside from the fact that N-2 is a parent common to both. The other common feature is that both crosses involve one clearly neoplastic line: N-1, a "high" cancer line, in the first cross, and Py 198-1, in the second cross. If one recalls that, according to recent work, the cell surface may be characteristically disorganized in neoplastic cells and, if one assumes that cell fusion depends on surface properties of the cells, it appears possible that this common feature of our two crosses is a significant one.

3. Lastly it may be recalled at this point that, according to recent work of Roizman,[10] polykaryocytosis, so frequent in tissue cultures, often results from virus infection, and that it consists essentially of successive cell fusions occurring between virus-infected and noninfected cells, a fact ascribed precisely to the alteration of the cell surface by the infecting virus. Py 198-1 is, of course, a virus-converted, neoplastic cell. The mechanism of the so-called "spontaneous," *in vitro* cancerization of line N-1 is not known, and the fact that no virus is known to have produced the neoplastic transformation of this line, derived from a normal mouse cell, or that no known virus is now released by cultures of N-1, does not preclude the possibility of viral origin of its neoplastic character.

Thus it remains possible that the basis of the observed hybrid formation is an event related, at least in terms of the primary mechanism, to the phenomenon of polykaryocytosis. In fact, since Roizman's experiments thus far have involved only virus-infected and noninfected cells of the same line, thus making the karyological detection of nuclear fusion products (indistinguishable in this case from 2*s* nuclei) impossible, one may wonder whether hybridization is not a frequent phenomenon in his experimental material or, putting it another way, if the incidence of hybridization could not be increased by the very treatments which promote polykaryocytosis.

The preceding remarks all have a bearing on the puzzling question of the mechanism of "mating." No doubt, much more insight into this process would be gained if we knew something about its quantitative, kinetic aspects. What do we know about the frequency of the phenomenon? All we can say at the present time is that, in the N-1 + N-2 combination, it cannot be an exceedingly rare event, because, as stated earlier, hybrid cells were detected in all four independent mixed cultures of our first experimental series. We may add here that we also succeeded in crossing several subclones of N-2 with line N-1. This does not tell us anything, however, about the absolute frequency of the unit mating event, nor about the number of these unit events that occurred

in a given mixed culture prior to the detection of the hybrid cells. To determine these values, i.e., to quantitate our system, we have to make it into a selective one; this, in turn, requires the introduction, into our mating cell lines, of selective markers. This is part of our program. If it were successful, we could apply to our system one of the quantitative techniques familiar to the microbial geneticists, for example, prototroph selection.

Meanwhile the only evidence on these points is indirect and comes from the consideration of the frequency distribution of the karyotypes of hybrid cells in the mixed cultures in which they first appeared. It will be seen that inferences from these remain inconclusive on some major points. They will be quoted here to show the nature of the problems and of the difficulties we are facing. On the other hand, the comparison of the distributions of hybrid karyotypes (established as early as was practically possible after their detection) with those established at different points in time, in pure cultures (i.e., after the elimination of the parental types), will provide the still fragmentary evidence we have collected during the past year on another question, important from the point of view of the phenomenon of somatic cell mating as a tool for the study of gene recombination. It is a question which we are systematically asked by geneticists whom we tell about our results: Is the formation of hybrids followed by segregation?

We may point out that, in our opinion, some degree of segregation in newly formed hybrids of high ploidy was to be expected on two grounds: (1) the analogy of this situation with that in the parasexual cycle, as described by Pontecorvo[3] in fungi, and (2) the well known fact that, in neoplastic tissues and *in vitro* cultures, the formation of tetraploid cells is usually followed by some degree of segregation. In both cases the basic mechanism is accidental loss of chromosomes.

In presenting our data on the frequency distribution of karyotypes, we must emphasize that, unless stated otherwise, we are going to describe a purely quantitative picture, as though the only changes occurring in time were changes in chromosome numbers. This should not be taken to mean that we are unaware of the possibility of the occurrence of undetected qualitative changes as well.

We shall start from our first hybrid, the already mentioned H 109t. The distribution of the karyotypes in the parental lines N-1 and N-2 is shown by the two upper histograms of Figure 7. It can be seen that each line is characterized by a rather clear modal number.

The distribution of karyotypes of hybrid H 109t, based on 50 mitoses studied between March 14 and May 23, i.e., during the period of their rise from the approximately 10 percent level (at the moment of their detection in this culture) to the 100 percent level, is shown by the upper, black histogram of Figure 8. The inverted, white histogram, below the abscissa, represents the distribution expected on the basis of multiple random matings between all karyotypes present in the two parental populations. There is a rather surprisingly good agreement between the distribution expected on this basis and the

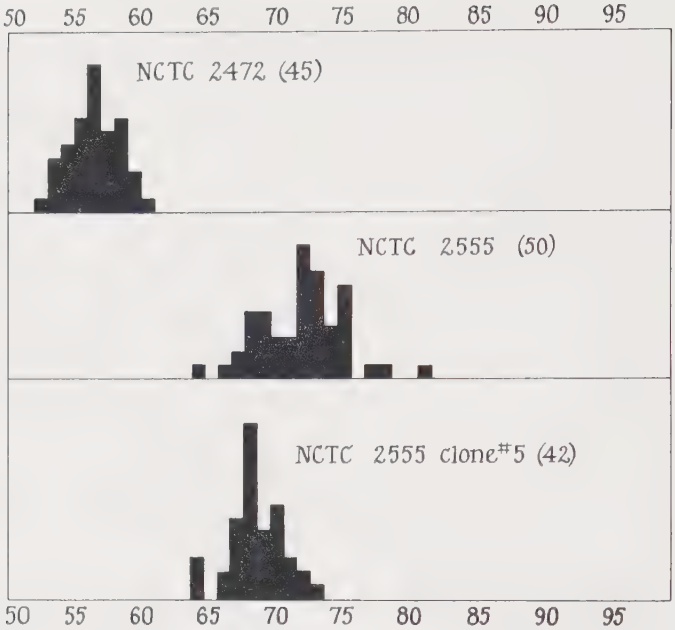

Fig. 7.—Distributions of number of chromosome arms in *s* cells of three cell lines. Number of analyzed cells in parentheses.

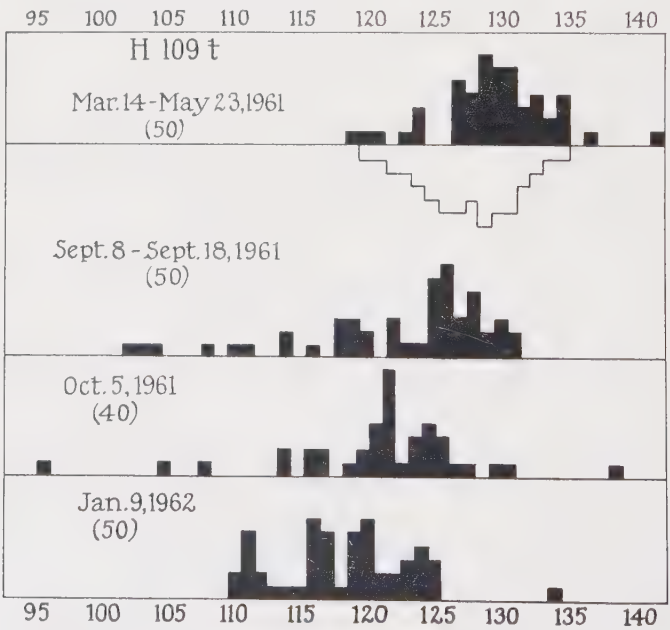

Fig. 8.—Distribution of numbers of chromosome arms in hybrid H-109t at various times.

distribution actually observed, the mean of the chromosome number of the hybrids being only slightly above the sum of the means of chromosome numbers of the two parental lines.

Thus, these results of our first experiment do not contradict (and, in fact, formally, lend strong support) to the hypothesis of multiple, random matings as a source of the hybrid population.

There is, however, some difficulty in accepting such a view (a) if one thinks of the fact that the coincidence between the expected and observed distributions implies not only random mating, but absence of any selection between the hybrid cells of various karyotypes as well; and (b) if from this purely formal aspect, one turns to the precise "case history" of H 109t. Then one is bound to ask why the matings, which on the panmictic (random mating) hypothesis must have been rather numerous during the indicated period, have not been observed during the preceding two months. The answer may simply be that the frequency of hybrids during this period was too low for detection within the small sample of analyzed metaphases.

It is of course possible that the hybrid population originated from rather rare matings (if not from a single mating event) between, say, modal cells of the two parental lines; and that the karyotype distribution revealed by the analysis of the first 50 mitoses corresponds to variations which occurred during the multiplication of the hybrid cell. Such variations are expected to occur indeed, but such an interpretation implies that the very good agreement between the two histograms of Figure 8, representing the expected and observed distributions, is purely accidental, and we find this somewhat difficult to believe.

Let us consider now the other three histograms of Figure 8 which describe the distributions of karyotypes in the pure hybrid culture H 109t during the following ten months. It can be seen that already in September (i.e., roughly four months after culture H 109t became practically a pure hybrid population) the originally "panmictic" distribution has changed into another one, characterized by lowered mean and mode and by increased variation. The progression of this trend is visible in the next, October distribution, while the January histogram seems to show a tendency to stabilization at a new equilibrium, with a mean of 118, instead of 130 chromosome arms.

Thus, during the ten months of study at least, there was an undeniable continuous drift which we consider to be proof of segregation.

Let us now consider another hybrid, I-5. This hybrid results from the cross of the same line N-1 with a clone (#5) isolated from our N-2 line. As shown by the lower histogram of Figure 7, clone 5 is a low clone of N-2; it presumably originated from a cell from the left end of the N-2 distribution. The modal number of chromosome arms in clone 5 is 68 as compared with 72 in line N-2. It must be emphasized, moreover, that we know that some chromosomal rearrangements had occurred in this clone.

A corollary of the "panmictic" hypothesis is that such a cross should give a hybrid karyotype distribution with a lower mode than that of the first N-1 $\times$ N-2 cross. This is indeed what happened and can be seen in the upper

histogram of Figure 9: the modal number of chromosome arms in hybrid I-5 is 121 as compared with 129 of hybrid H 109t. On the other hand, the comparison of the observed distribution with that predicted on the random mating hypothesis, shows that the former one is considerably to the left of the latter, i.e., in this case, we do not find the surprising agreement between observation and expectation that was found for hybrid H 109t. Although the hybrid cells in this cross were also detected (at the 10 percent level) two months after the beginning of the experiment, and although the 50 mitoses comprised in the first distribution were analyzed at that moment, we cannot safely conclude that this result contradicts the panmictic hypothesis for the following reason. The karyotype distribution in clone 5 was established on a pure culture of this line and *not* in the mixed culture. Therefore, we cannot say that there was no random mating for it is equally possible that the karyotype of clone 5 cells has shifted *after* they were introduced into the mixed culture, but *prior* to mating.

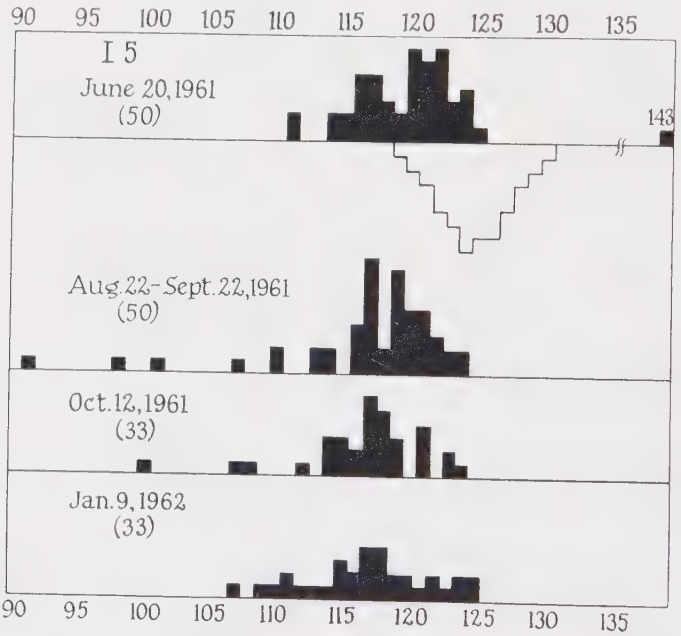

Fig. 9.—Distribution of numbers of chromosome arms in hybrid I-5 at various times.

Whether there was a drift of the hybrid karyotype at this stage or not, a slight one occurred later, as shown by the three other histograms of Figure 9. It can be seen that, starting from a mode of 121 chromosome arms, I-5, in the course of the next several months, drifted to a mode of 117 or 118 chromosome arms. Thus, in this case too, there is evidence for chromosome segregation.

We now turn to our hybrid M-109 from the cross of line N-2 with line Py 198-1. The upper histogram of Figure 10 shows the distribution of karyotypes of 24 metaphases analyzed up to the moment when the hybrids represented 10 percent of the population. The distribution expected on the basis of panmixia is not given in this case because we did not have sufficient information on the Py 198-1 distribution. However, something concerning the number of mating events can be inferred from the following observation. The black squares on the histogram indicate the karyotypes of the six first hybrid cells analyzed at the moment of their first detection at the 1 p. 1,000 level. Their grouping is remarkable and, on the basis of chance alone, could be expected to occur only once in 700 trials. We regard this as a strong indication that, in this case at least, the initial mating event was probably unique and involved modal cells. Whether the variation observed among the later 18 mitoses is secondary and due to segregational drift alone, or to additional matings as well, is difficult to decide. The only assumption that may be safely excluded is that this heterogeneity is entirely due to additional matings, for some chromosome numbers are distinctly too low (this is confirmed also by the lowest numbers of metacentrics.)

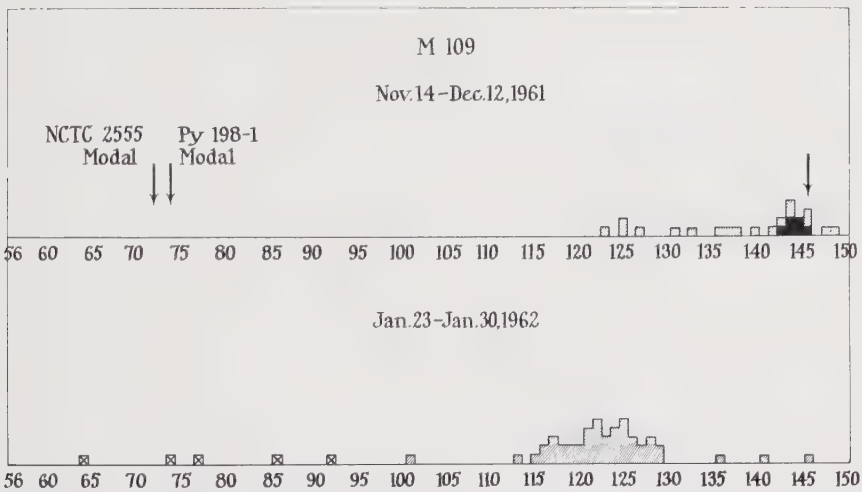

Fig. 10.—Distribution of numbers of chromosome arms in hybrid M-109 at various times.

The further evolution of M-109 hybrids (lower histogram, Figure 10) is characterized by a rapid segregation, resulting in both a considerable lowering of the mode (from 143 to 122 chromosome arms) and a wide range of variation, including the appearance of rare "very low" karyotypes. (These are marked distinctively to indicate that their very low frequency is not correctly reflected in this histogram.)

We shall now try to sum up the tentative conclusions from the presented data.

1. The first thing we wish to emphasize is that the occurrence of hybridization has now been observed in two combinations (not counting sublines) and substantiated by the isolation of hybrid clones.

2. Concerning the mating process itself, our findings on hybrid M-109 are not inconsistent with the hypothesis that the hybrid population arose from a single mating event involving modal cells of the two parental lines, followed by rapid segregation. Whether this mating pair remained unique is not certain.

3. *A priori,* a similar interpretation should therefore be applicable to hybrid H 109t. However, the remarkable coincidence of the observed karyotype distribution with that predicted on the basis of multiple, random matings must then be regarded as purely accidental. We hesitate to consider the problem as thus settled, and wonder whether cultural conditions have not been especially favorable for mating at some point in the history of our culture H 109t.

4. Concerning the occurrence of segregation, all that can be said at the present time is that, as expected, this process does occur. It is interesting to observe that, in our hybrids, this segregation proceeded faster the higher the initial number of chromosomes (Table 2); that the karyotypes of hybrids H 109t and I-5 at present tend to stabilize at a new equilibrium; and that this new numerical equilibrium seems to be quasi-identical for these two hybrids in spite of the initial quantitative and qualitative differences. Future analysis will show whether this holds for hybrid M 109 also.

TABLE 2.—*Evolution of the Karyotypes of Hybrids H-109t, I-5, and M-109*

Time Since First Distribution (months)	H–109t		I–5		M–109	
	Chromosome No. (mean)	Loss (%)	Chromosome No. (mean)	Loss (%)	Chromosome No. (mean)	Loss (%)
0	113.5		105.9		124.7	
2					110.3	11.6
3	106.9	5.9	103.6	2.2		
4	105.9	7.7	103.6	2.2		
7	103.4	8.3	103.8	2.0		

As stated earlier, the solution of the problems raised in this paper would, *a priori,* be greatly facilitated by appropriate markers. The importance of marker genes is obvious but it is not certain that it cannot be overestimated. Informal reports have reached us that crossing of somatic cells, using marked cell lines, has been attempted in several laboratories, but thus far no success has been recorded. It may be a valid question whether our success, if it is not based on the use of a unique mater line, is not due precisely to the use of the otherwise inefficient, purely karyological detection technique.

REFERENCES

1. Pontecorvo, G.: Methods of Microbial Genetics in an Approach to Human Genetics. Brit. M. Bull. *18*:81–84, 1962.
2. Ryan, F. J.: Applicability of Approaches of Microbial Genetics to Characterization of Mammalian Cells. Univ. Michigan M. Bull. *28*:267–276, 1962.
3. Pontecorvo, G.: *Trends in Genetic Analysis*. New York: Columbia University Press, 1958.
4. Lederberg, J.: Genetic Approaches to Somatic Cell Variation: Summary Comment. J. Cellul. Physiol. *52*(Suppl. 1):383–401, 1958.
5. Sorieul, S., and Ephrussi, B.: Karyological Demonstration of Hybridization of Mammalian Cells *in vitro*. Nature *190*:653–654, 1961.
6. Ephrussi, B., and Sorieul, S.: Nouvelles Observations sur l' Hybridation *in vitro* de Cellules de Souris. C. rend. Acad. sc. *254*:181–182, 1962.
7. Barski, G.; Sorieul, S.; and Cornefert, F.: Production dans des Cultures *in vitro* de deux Souches Cellulaires en Association, de Cellules de Caractère "Hybride." C. rend. Acad. sc. *251*:1825–1827, 1960.
8. Sanford, K. K.; Likely, G. D.; and Earle, W. R.: The Development of Variations in Transplantability and Morphology Within a Clone of Mouse Fibroblasts Transformed to Sarcoma Producing Cells *in vitro*. J. Nat. Cancer Inst. *15*:215–230, 1954.
9. Sanford, K. K.; Merwin, R. M.; Hobbs, G. L.; and Young, J. M.: Clonal Analysis of Variant Cell Lines Transformed to Malignant Cells in Tissue Culture. J. Nat. Cancer Inst. *23*:1035–1059, 1959.
10. Roizman, B.: Polykaryocytosis Induced by Viruses. Proc. Nat. Acad. Sc. *48*:228–234, 1962.

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